IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Hans Klingemann	)	
	)	
Serial No. 10/008,955	)	**NATURAL KILLER CELL LINES AND METHODS OF USE**
	)	
Filed: December 7, 2001	)	
	)	
Art Unit: 1644	)	
	)	
Patent Examiner: Ronald B. Schwadron	)	
	)	
Attorney Docket No. 06-129 PCT/US/CIP	)	
	)	
Confirmation No.: 5420	)	

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March 23, 2010

AMENDED APPEAL BRIEF PURSUANT TO 37 C.F.R. § 41.37

Applicant regrets that the Amended Appeal Brief submitted on December 9, 2009, was found defective for the reasons set forth in the Notification of Non-Complaint Appeal Brief dated February 24, 2010. The amended Appeal Brief was complete in most all respects but, according to the Notification of Non-Compliant Appeal Brief, (i) disclosed applications that are not under appeal or the subject of an interference; (ii) did not indicate in the Evidence Appendix where in the record the Arai et al. reference was entered by the Examiner; (iii) did not indicate in the

Evidence Appendix where in the record cite (3) evidence was entered by the Examiner; or (iv) address the Tam et al. or Santoli et al. (Cancer Res.) or Cesano et al. references cited in the Brief.

Accordingly, Applicant hereby amends the Amended Appeal Brief to comply with the requirements set forth in 37 C.F.R. § 41.37(c) to (i) remove from Sections II and X disclosed applications that are not under appeal or the subject of an interference and (ii) to include evidence in the Evidence Appendix and to provide copies of same. Cite (3) (ATCC Culture Requirements) has been removed from the Evidence Appendix and Footnote 2, which referenced ATCC Culture Requirements, has been removed from the Brief.

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TABLE OF AUTHORITIES

Cases

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I. REAL PARTY IN INTEREST

The subject application has been assigned to ZelleRx Corporation who is the real party in interest.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III. STATUS OF CLAIMS

Claims 20, 22, 26, 27, and 30 are currently pending. Claims 1-19, 21, 23-25, 28, 29, and 31 are withdrawn from consideration. For convenience, the complete text of the claims is attached hereto in the Claims Appendix in Section VIII hereto. Claims 20, 22, 26, 27, and 30 were finally rejected and are on appeal:

(a) Claims 20, 22, 26, 27, and 30 are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 30-35, 46, 48, 50, and 53 of co-pending U.S. Appn. No. 10/701,359 ("the '359 Application"). Applicant indicated in the Request for Continued Examination filed on October 15, 2008 that a Terminal Disclaimer will be filed upon recognition of allowable subject matter.

(b) Claims 20, 22, 26, 27, and 30 are rejected pursuant to 35 U.S.C. § 103(a) as being unpatentable over Gong et al., *Leukemia* 8:652-658, 1994 ("Gong et al.") in view of U.S. Patent No. 5,272,082 to Santoli et al. ("Santoli et al.").

IV. STATUS OF AMENDMENTS

Applicant filed an amended claim set with the Request for Continued Examination filed on October 15, 2008[1]. Applicant has not filed any amendment subsequent to the Final Office Action mailed on March 24, 2009.

Applicant filed the Notice of Appeal on September 15, 2009.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Independent claim 20 is directed to a method of treating a pathology *in vivo*. See the '955 Application as originally filed, pg. 1, lines 8-10 (hereinafter, "the '955 Appn., __:__"); see also the '955 Appn. 4:24-26; 5:9-11; 8:9-26; 13:11-20; 15:29-16:5; 16:19-24; 23:24-24:2; 26:18-22; 35:12-25; 39:3-10; 39:19-22; 41:26-42:5; 43:22-29; 44:12-28; 46:24-27; Table 1; Table 5; Table 6; Figure 8; and Figures 11-13). The method is carried out in a mammal. See the '955 Appn., 8:10; 13:16; 18:23-26; 23:24-25; 41:26-30; and 44:1-46:27. Applicant developed a unique cell line identified as NK-92 and available from American Type Culture Collection (ATCC) as Deposit No. CRL-2407. The claimed method comprises the step of administering to the mammal a medium comprising NK-92 cells. See the '955 Appn., 1:10; 8:11; 13:17-18; 14:4-5; 16:11-25; 23:25; and 25:10-12.

Dependent claim 22, which depends from claim 20, is directed to a method wherein the pathology is a cancer. See the '955 Appn., 1:9; 4:25; 5:10; 13:11-13; 23:23-24:2).

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[1] The Request for Continued Examination was refiled on January 15, 2009 in the Response to Notice of Non-Compliant Amendment to correct the status identifiers of claims 23 and 31 that were improperly labeled as "withdrawn – previously presented" rather than "withdrawn."

Dependent claim 26, which depends from claim 20, is directed to a method wherein the cells are administered to the mammal intravenously. See the '955 Appn. 8:21-22; 23:26-27; 23:29-24:2). The mammal is a human. See the '955 Appn. 8:21-22.

Dependent claim 27, which depends from claim 21, further comprises the step of administering to the mammal a cytokine that promotes the growth of the NK-92 cells. See the '955 Appn., 8:21-24; 24:13-23).

Dependent claim 30, which depends from claim 22, is directed to a solid tumor cancer. See the '955 Appn., 23:29.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1) The rejection of claims 20, 22, 26, 27, and 30 pursuant to 35 U.S.C. § 103(a) as being unpatentable over Gong et al. in view of Santoli et al. is being appealed.

2) The rejection of claims 20, 22, 26, 27, and 30 on the grounds of nonstatutory obviousness-type double patenting as being unpatentable over claims 30-35, 46, 48, 50, and 53 of co-pending Application No. 10/701,359 is not being appealed. A terminal disclaimer will be submitted upon an indication of allowability of the pending claims.

3) The Examiner's refusal to enter the Substitute Specification on the grounds that it does not conform to 37 C.F.R. § 1.125(b), (c) because it allegedly contains new matter is not being appealed. Applicant will cancel the alleged new matter upon an indication of allowability of the pending claims.

4) The objection to the amendment filed on October 15, 2008 pursuant to 35 U.S.C. § 132(a) on the grounds that new matter is introduced into the disclosure is not being appealed.

As set forth above, Applicant will cancel the new matter upon an indication of allowability of the pending claims.

VII. ARGUMENT

A. Introduction

This appeal is based on Applicant's belief that the Examiner has failed to establish a *prima facie* case of obviousness on which to base the current rejections of the claims pursuant to 35 U.S.C. § 103(a). The Examiner's rejection is erroneously premised on the combination of two references, namely Gong et al. in view of Santoli et al., despite the fact that the combination of references fails to teach or suggest Applicant's method of treating a pathology *in vivo* comprising the step of administering to the mammal a medium comprising NK-92 cells. Further support for the lack of obviousness is found in the Declaration of Hans Klingemann, M.D., Ph.D., pursuant to 37 C.F.R. § 1.132 (hereinafter, "Klingemann Decl."), the sole inventor of the NK-92 cells disclosed in the '955 Application and a skilled artisan in the fields of translational research, transplantation biology, and tumor immunology. Klingemann Decl., ¶ 14.

Gong et al. disclosed the NK-92 cell line, an immortal cell line originally obtained from peripheral mononuclear cells of a fifty-year-old male patient having non-Hodgkin's lymphoma. Klingemann Decl., ¶ 21; '955 Appn., 14:4-5. At the time that the NK-92 cell line was discovered, the inventor thought that the cell line provided a suitable model to study the biology of NK-cell and activated NK-92 cells. Klingemann Decl., ¶ 22. Gong et al. merely set out to characterize the NK-92 cell line for use as a research tool. Gong et al., Abstract.

Further research with the NK-92 cell line revealed surprising and unexpected results, and it is these further inventions that are claimed in the '955 Application. '955 Appn., 43:22-29. Of

particular surprise was the finding that NK-92 cells have cytolytic activity *in vitro* and tumor-inhibiting activity *in vivo*. '955 Appn. 43:22-29. Specific data demonstrating the cytoxic activity of the NK-92 cells are set out in Tables 5 and 6 and Figure 9 of the '955 Application, reproduced below.

Table 5. Cytotoxicity of NK-92, T-ALL104 and YT Clone to Patient-Derived Leukemic Cells[a]

Patient	Disease Status	Blast (%) in Sample	Cytotoxic Sensitivity		
			NK-92	TALL-104	YT
AML					
1 M4□	Relapse	PB (66%)	++++++	+++++	-
2 (M1)	Relapse	PB (50%)	+++++	-	-
3 (M3)	Relapse	PB (50%)	+++ (++++)	+ (++++)	- (-)
4 (M4)	Refractory	PB (90%)	++ (++)	- (+)	- (-)
5 (M2)	New	BM (90%)	+++ (+++)	+ (+++)	ND
6 (M4)	New	BM (97%)	-	-	-
7 (M4)	New	PB (39%)	- (-)	- (++)	- (-)
8 (M3)	New	PB (55%)	- (++)	- (+++)	+ (-)
9 (M3)	New	BM (32%)	-	-	-
T-ALL					
1	Relapse	BM (98%)	++++++	-	-
2	Relapse	PB (85%)	++++++	- (-)	+++ (+++)
3	Relapse	PB (77%)	++++++	- (+)	- (-)
4	Relapse	PB (60%)	+++++	- (-)	+ (-)
5	New	BM (40%)	+++	-	-
6	New	BM (66%)	+++	-	-
B-Lineage-All					
1 ●	Relapse	BM (78%)	+++++	++++	-
2	New	BM (30%)	++++	ND	ND
3	Relapse	BM (75%)	+++ (++++)	+ (++++)	++ (++)
4	New	BM (97%)	++ (+++)	+ (+++)	- (-)
5	Relapse	BM (60%)	+ (+)	- (+)	- (-)
6	Relapse	BM (80%)	-	ND	ND
7	Relapse	PB (80%)	-	- (-)	-
8	New	BM (68%)	-	-	-
9	New	BM (33%)	-	- (+)	-

Patient	Disease Status	Blast (%) in Sample	Cytotoxic Sensitivity		
			NK-92	TALL-104	YT
10	Relapse	BM (87%)	-	- (++)	-
11	Relapse	BM (75%)	- (+++)	- (++++)	-
12	New	BM (30%)	-	-	ND
13	New	PB 90%)	- (+++)	- (+++)	ND
14	New	BM (81%)	-	-	ND
CML					
1	BC	PB (45%)	++++++	+++++	+++
2	AC	PB (22%)	++++++	++	-
3	BC	PB (93%)	+++++	+	-
4	CP	PB (15%)D	++++	+	-
5	CP	PB (8%)D	++ (++++)	ND	ND
6	CP	BM (12%)D	+ (+++)	+ (+)	ND
7	CP	BM (10%)D	+ (+++)	+ (++++)	ND
8	BC	PB (60%)	+	-	-
9	BC	BM (48%)	+	- (-)	-
10	CP	PB (21%)D	+ (++)	- (++++)	- (-)
11	CP	PB (11%)D	-	- (+++++)	- (-)

Notes and Abbreviations. a) Columns show results of chromium release assays at E:T = 9:1 after 4 h without parentheses, and (results after 18 h enclosed in parentheses); New: newly diagnosed; ND: none done; o: FAB classification; D:blast and promyelocyte; BM: bone marrow; PB: peripheral blood; I:B-ALL; BC: blast crisis; AC: accelerated phase; CP: chronic phase.

Table 6. Specific Lysis of Human Leukemia Cell Lines by Natural Killer Cell ClonesNK-92, TALL-104, and YT.

Target	Specific Lysis (%)								
	NK92			TALL-104			YT		
	Effector:Target Ratio								
	9:1	3:1	1:1	9:1	3:1	1:1	9:1	3:1	1:1
K562	94.1	91.2	82.1	88.5	85.2	72.5	34.2	28.2	18.4
HL60	87.9	75.3	79.6	43.0	16.0	6.9	2.1	1.1	1.5
KG1	64.6	53.8	43.7	2.7	0.5	0	0.1	0	0
NALM6	72.6	56.8	52.4	67.8	55.6	33.3	1.0	0.5	0
Raji	86.0	75.4	70.0	22.2	10.2	0.3	25.1	18.0	14.2
TALL-104	57.3	53.2	44.1	-	-	-	3.2	1.4	0.9
CEM/S	56.6	48.8	34.7	2.7	1.6	0.9	0.9	0.4	0.3
CEM/T	57.5	42.1	39.1	1.5	0.6	0.3	1.2	0.1	0.2

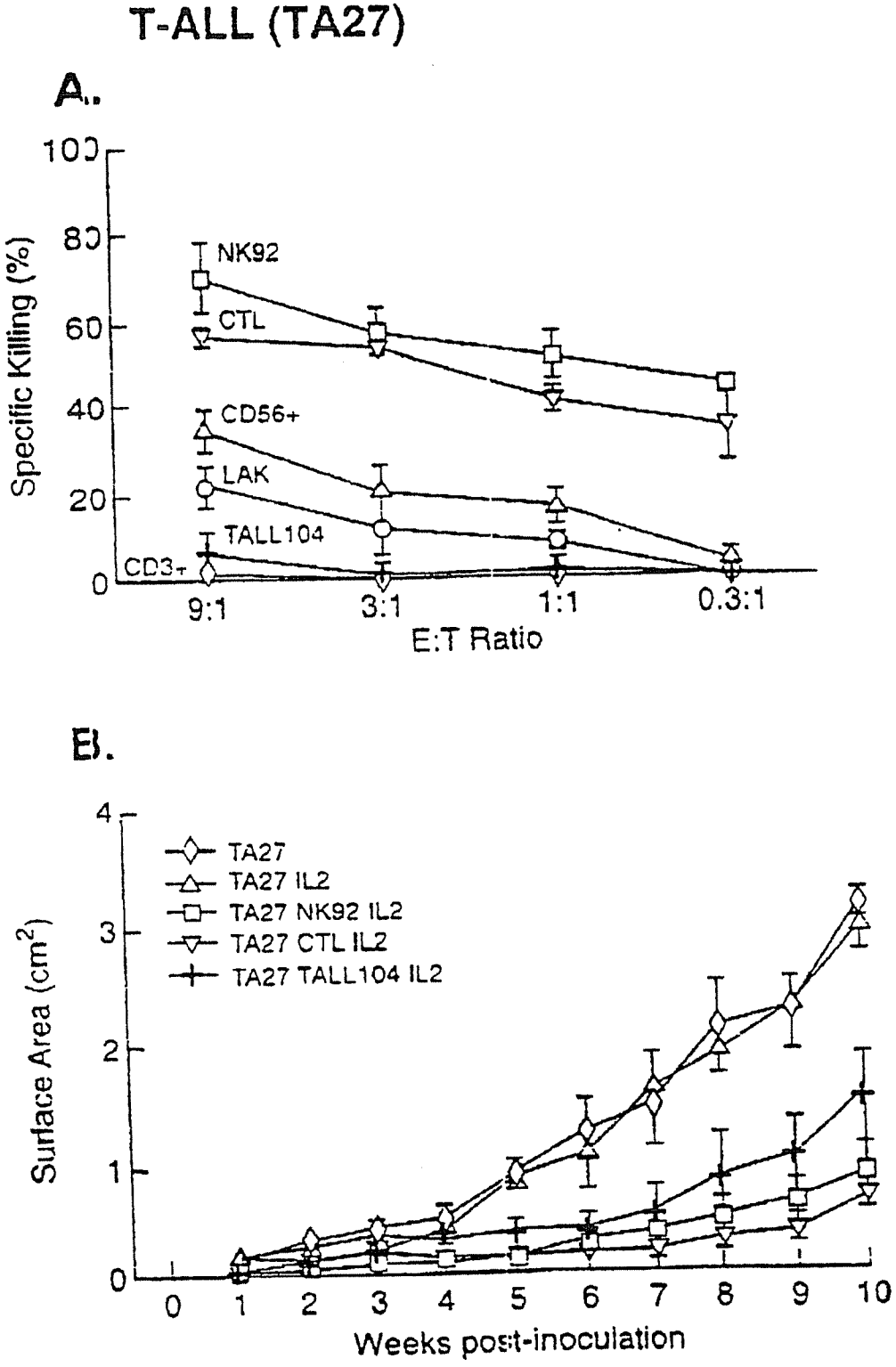
T-ALL (TA27)
A.
Specific Killing (%)
100
80
60
40
20
0
NK92
CTL
CD56+
LAK
TALL104
CD3+
9:1
3:1
1:1
0.3:1
E:T Ratio
B.
Surface Area (cm2)
4
3
2
1
0
TA27
TA27 IL2
TA27 NK92 IL2
TA27 CTL IL2
TA27 TALL104 IL2
0 1 2 3 4 5 6 7 8 9 10
Weeks post-inoculation

FIGURE 9

In contrast to the teachings of Gong et al., Santoli et al. teach "genetically modified cytotoxic T lymphoblastic leukemia cell lines (T-ALL), and uses of these cell lines in cancer therapy." Santoli et al., Abstract. There is absolutely no teaching or suggestion of Applicant's claimed method in either Gong et al. or Santoli et al., alone or in combination. As such, the Examiner's rejection of claim 20 and claims 22, 26, 27, and 30 depending therefrom is completely unsubstantiated. The rejection should be reversed.

B. Applicant's Claimed Method of Treating a Pathology *In Vivo*

Unlike anything shown in the combination of Gong et al. and Santoli et al., Applicant's independent claim 20 claims a method of treating a pathology *in vivo* by administering a medium comprising a particular line of NK-92 cells.

Dependent claim 22, which depends from claim 20, is for a method of treating a cancer.

Dependent claim 26, which depends from claim 20, provides that the NK-92 cells are administered to the mammal intravenously and that the mammal is a human.

Dependent claim 27, which dependents from claim 20, further comprises the step of administering to the mammal a cytokine that promotes the growth of the NK-92 cells.

Dependent claim 30, which depends from claim 22, limits the cancer to a solid tumor.

C. For the reasons explained herein, the Examiner cannot properly rely on the combination of Gong et al. and Santoli et al. to support a rejection of the claims pursuant to 35 U.S.C. § 103(a).

C. Independent claim 20 is not obvious over Gong et al. in view of Santoli et al. because claim 20 recites subject matter not shown or suggested by the cited prior art.

Pursuant to 35 U.S.C. § 103(a), the Examiner has finally rejected claims 20, 22, 26, 27, and 30 directed to a method of treating a pathology *in vivo* in a mammal comprising the step of administering to the mammal a medium comprising NK-92 cells (available from ATCC as Deposit No. CRL-2407) as being unpatentable over Gong et al. in view of Santoli et al.

Specifically, the Examiner alleges that

> Gong et al. teach use of NK-92 cells to lyse leukemic tumor cells. Gong et al. teach that said cells require IL-2 to function. Gong et al. does not in vivo use of NK-92 cells to treat cancer [sic]. Santoli et al. teach that lytic human derived cell lines can be used in vivo to treat disease or in preclinical in vivo studies. Santoli et al. teach that said cells are injected iv wherein injection utilizes a syringe and wherein the injected NK-92 cells would be adjacent to leukemic cells in the blood. Santoli et al. disclose that the cells can be administered with the cytokine IL-2. Santoli et al. teach that said cells can be modified to bind solid tumors. It would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to have created the claimed invention because Gong et al. teach use of NK-92 cells to lyse tumor cells, while Santoli et al. teach in vivo use of cytotoxic cell lines. One of ordinary skill in the art would have been motivated to do so because Santoli et al. teach that lytic human derived cell lines can be used in vivo to treat disease or in preclinical in vivo studies.

Final Office Action, ¶ 8. Applicant disagrees for at least the reasons set forth below.

1. Disclosure of Gong et al.

Gong et al. established the existence of the immortal cell line, NK-92, and set out to characterize the NK-92 cell line for use as a research tool, concluding that the NK-92 cell line "may provide a suitable model to study certain aspects of [Natural Killer/Activated Natural

Killer] cell biology." Gong et al., 658; see also Gong et al., Abstract; see also Klingemann Decl., ¶ 22. Gong et al. also partially characterized the phenotype of NK-92 cells. Gong et al., 654. The NK-92 cell line was established from peripheral blood mononuclear cells of a fifty-year-old male patient who was diagnosed with an aggressive LGL lymphoma in 1992. Klingemann Decl., ¶ 21; '955 Appn., 14:4-5. While Gong et al. provide data that suggest that NK-92 cells kill K562 and Daudi cells in a chromium release assay (see Gong et al., 654 and Fig. 4), all experiments were performed *in vitro*. There is absolutely no teaching or suggestion in Gong et al. of a method of treating a pathology *in vivo* in a mammal comprising the step of administering to the mammal a medium comprising NK-92 cells, as in Applicant's independent claim 20.

The Examiner incorrectly states that "Gong et al. teach use of NK-92 cells to lyse leukemic tumor cells." Final Office Action, ¶ 8. Rather, Gong et al. teach that NK-92 cells demonstrated cytotoxicity against two human leukemic cell lines. Gong et al. simply do not teach that NK-92 cells are capable of lysing various tumor cells, including other leukemic tumor cells, of different origin or type. Klingemann Decl., ¶ 24a. As such, there is simply no teaching, suggestion, or motivation in Gong et al. that would lead one skilled in the art to use the NK-92 cell line *in vivo* to lyse tumor cells or as a cancer treatment, much less successfully reduce such a use to practice as a method of treating mammals. Id., ¶ 24c. Simply because a cell line is developed or established in the laboratory does not mean that there is an expectation of success for utilizing that cell line in a clinical setting. Certainly that was not the expectation with the NK-92 cell line because even the inventor did not initially recognize the importance or utility of the NK-92 cells in a clinical setting. Id.

2. Disclosure of Santoli et al.

Santoli et al. teach genetically modified cytotoxic T lymphoblastic leukemia (T-ALL) 104 and 107 cell lines and uses of these cell lines to treat cancer, both *in vivo* and *ex vivo*. Santoli et al., Abstract, 10:30-60. NK-92 cells are not disclosed by Santoli et al., nor is the use of NK-92 cells described. In fact, Santoli et al. do not provide a teaching, suggestion, or guidance with respect to any cell line other than T-ALL cells. In particular, Santoli et al. do not consider, teach, suggest, or provide guidance to NK-92 cells.

The Examiner misconstrues the teaching of Santoli et al. when, citing to Column 10 of Santoli et al., he states that "Santoli et al. teach that lytic human derived cell lines can be used in vivo to treat disease or in preclinical in vivo studies." Final Office Action, ¶ 8. Rather, contrary to the Examiner's position, Santoli et al.'s teaching is limited to the use of "this invention," (i.e., T-ALL cells), not to all lytic human derived cell lines. The Examiner's conclusion is simply overly broad.

3. Santoli et al. is not relevant to Applicant's claimed method or combinable with Gong et al. because Santoli et al. disclose T-ALL cells which are structurally and functionally distinct

As set forth in the table below, the NK-92 cell line taught in Gong et al. or claimed by Applicant in independent claim 20 is structurally and functionally distinct from Santoli et al.'s T-ALL cell lines. Klingemann Decl., ¶ 28. As emphasized in Dr. Klingemann's declaration, know-how with respect to one cell line cannot automatically be transferred or applied to another cell line, even when the cells actually are closely related (which is not the case with NK-92 cells and T-ALL cells), including with respect to culture conditions, requirements for growth factors such as IL-2, survival and signaling patterns following adoptive transfer, ability to migrate to

tumor sites, sensitivity to chemotherapeutic agents, response to staining with vital dyes, ability to maintain their cytotoxic activity following radiation, and susceptibility to gene transfer. Id., ¶ 27. Furthermore, the know-how required to use a specific cell line as a method of treatment cannot automatically be transferred or applied to another cell line and is dependent on the distinguishing characteristics of each cell line. Id. Simply because one cell line has a specific utility does not mean that other closely related cell lines will have the same utility. Id. Each must be proven independently and the specific conditions necessary for successful results, including treatment, determined. Id.

Comparison of NK-92 cells to T-ALL cells

NK-92 Cells	T-ALL Cells
Derived from patient with aggressive LGL lymphoma	Derived from patient with T lymphoblastic leukemia
Originate from natural killer cells	Originate from T-cells
Do not require antibody stimulation in culture	Require antibody stimulation in culture
Maintain cytotoxicity and function after irradiation	Lose some cytotoxicity after irradiation
Have higher cytotoxicity than T-ALL cells	Have lower cytotoxicity than NK-92 cells

a. NK-92 cells and T-ALL cells were derived from different disease categories

The T-ALL cell line was derived from a patient with T lymphoblastic leukemia (T-ALL) (Santoli et al., 2:41-43), whereas the NK-92 cell line was derived from a patient with an aggressive LGL lymphoma. Klingemann Decl., ¶ 21. Leukemia and lymphoma are in different

disease categories and the cells derived therefrom are different cell lineages. Klingemann Decl., ¶ 28a. As such, the cell lines each have unique characteristics in culture and in undergoing proliferation. Id. As the inventor of the NK-92 cell line noted in his declaration, one skilled in the art would therefore assume that these two cell lines are different and that conclusions with respect to one of the cell lines cannot be drawn to the other cell line. Id.

b. NK-92 cells and T-ALL cells have different origins

T-ALL cells are of T-cell origin, are CD3-positive (a specific T-cell marker), CD8-positive, rearrange and express the T-cell receptor, are TCRαβ-positive, and are characterized by specific chromosomal translocations. *See* Santoli et al., 1:68, 2:14, and 4:27; *see also* Klingemann Decl., ¶ 28b. In addition, T-ALL cells lack natural cytotoxicity receptors such as NK-44 receptors that are found on NK-92 cells. In contrast, the NK-92 cell line is derived specifically from natural killer cells, making it a true NK cell line. Klingemann Decl., ¶ 28b. NK-92 cells are CD3-negative, CD8-negative, do not express or rearrange the T-cell receptor complex (TCR), and have different chromosomal rearrangements than T-ALL cells. Gong et al., 657-658; Klingemann Decl., ¶ 28b. As such, one cannot infer the behaviors, transfectability, or cytotoxic mechanisms of NK-92 cells from those of T-ALL cells because the cells have different phenotypes. Klingemann Decl., ¶ 28b.

c. NK-92 cells and T-ALL cells have different culture requirements

The culture for NK-92 cells is different from the culture for T-ALL cells. *See* Klingemann Decl., ¶ 28c. While T-ALL cells require antibody stimulation with CD2 or CD3 (a specific T cell marker) antigens to express IFN-γ, TNF-α, and GM-CSF (Santoli et al., 2:18, 47),

NK-92 cells do not require such antibody stimulation, but rather release these cytokines in response to stimulation by IL-2. Gong et al., 654; *see also* Klingemann Decl., ¶ 28d. Specifically, when NK-92 cells are cultured in α-minimum essential medium (α-MEM), the American Type Culture Collection (ATCC; Manassas, VA) recommends the media be supplemented with, among other things, 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, 100-200 U/ml recombinant IL-2 (otherwise the cells die after 72 hours), and most surprisingly, a large proportion (25%) of two sera: 12.5% horse serum and 12.5% fetal bovine serum (FBS). In earlier passages, hydrocortisone is necessary. The cell density in culture is critical, and must be regularly checked and regulated by medium changes. The medium formulation, IL-2 concentration, serum concentration and cell density must be carefully regulated throughout the culture period. The culture of these cells are in stark contrast to other well-established cell lines (or even hybridomas), such as Madin-Darby Canine Kidney (MDCK) cells, which can thrive in simple MEM with 5% (FBS) and 2mM L-glutamine, 10mM N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), and sub-culturing once or twice a week.

d. <u>NK-92 cells are more stable than T-ALL cells</u>

Additionally, NK-92 cells are more stable than T-ALL 104 cells. Tam et al. (Hum. Gene Ther., 10:1359-1373, 1999) have shown that NK-92 (both wild-type and transfected cells) cells require > 500 Gy to suppress proliferation, while Santoli et al., Cancer Res., 56:3021-3029, July 1996, reported that T-ALL 104 cells require 40 Gy irradiation to suppress proliferation. Additionally, NK-92 cells maintain cytotoxicity and function even after irradiation, while T-ALL cells lose some cytotoxicity when irradiated. Klingemann Decl., ¶ 28e.

It has been reported that the standard treatment protocol for clinical trial in dogs required that the dogs be immunosuppressed using CsA, an immunosuppressive drug, starting the day before T-ALL 104 injections began and continuing through the first two weeks of T-ALL 104 injections. Santoli et al., Cancer Res., 56:3021-3029, July 1996. In contrast, NK-92 cells do not require supplemental immunosuppression. Klingemann Decl., ¶ 28f.

e. NK-92 cells have higher cytotoxic activity than T-ALL cells

Notably, comparative studies of NK-92 cells and T-ALL 104 cells further demonstrate that these cell lines are functionally quite different, with NK-92 cells having significantly higher cytotoxic activity than T-ALL 104 cells. For example, many hematological cancers are susceptible to killing by NK-92 cells, whereas these cancers are mostly resistant to lysis by T-ALL 104 cells. Klingemann Decl., ¶ 31. In fact, data disclosed in the '955 Application demonstrate that NK-92 cells are more cytolytic than T-ALL 104 cells or YT cells. See '955 Application, 35:11-36:20; 39:3-22; 44:11-28; Tables 5 and 6, Fig. 9. Even the inventor of the NK-92 cell line has indicated that the results demonstrating the superiority of the NK-92 cell line were surprising. Klingemann Decl., ¶ 33.

f. Summary

For at least these reasons, NK-92 cells are structurally and functionally different from the T-ALL cells disclosed by Santoli et al. One skilled in the art would therefore assume that conclusions with respect to one of these cell lines cannot be drawn to the other cell line. Klingemann Decl., ¶ 28a.

4. Applicant's method of treating a pathology as set forth in claim 20 is patentable over Gong et al. in view of Santoli et al. because Gong et al.

merely established the NK-92 cell line and its phenotype while Santoli et al. is only relevant to T-ALL cells

Applicant disagrees with the Examiner's rejection of independent claim 20 for obviousness over Gong et al. in view of Santoli et al. because the combination of references fails to teach or suggest each and every element of Applicant's claimed method of treating a pathology *in vivo* by administering to the mammal NK-92 cells.

The Examiner has the burden pursuant to 35 U.S.C. § 103 to establish a *prima facie* case of obviousness. In re Piasecki, 745 F.2d 1468 (Fed. Cir. 1984). To establish a *prima facie* case of obviousness, the Examiner must show: (i) a suggestion or motivation in the prior art, either from the references themselves or from generally available knowledge, for a person skilled in the art to choose the prior art reference or to combine the teachings of the references; (ii) a reasonable expectation of success; and (iii) that the reference or combination of references teach or suggest all of the claim limitations. See M.P.E.P. §§ 2141-2142; see also KSR Int'l Co. v. Teleflex, Inc., 127 S. Ct. 1727, 1741 (2007) (refusing to reject the use of teaching, suggestion, or motivation as a factor in the obviousness analysis because most inventions rely upon building blocks "long since uncovered, and claimed discoveries almost of necessity will be combinations of what, in some sense, is already known"). One court has noted that "[t]he KSR opinion only focused on the Federal Circuit's strict use of the [Teaching, Suggestion, Motivation] test in performing the obviousness analysis; it did not mention or affect the requirement that each and every claim limitation be found present in the combination of the prior art references before the analysis proceeds." Abbott Labs. V. Sandoz, Inc. 2007 U.S. Dist. Lexis 38216, *11 (N.D. Ill. 2007). Thus, KSR does not affect the Federal Circuit's holding that it is improper for the

Examiner to use the applicant's invention as a blueprint to hunt through the prior art for the claimed elements and then combine them as claimed. See, e.g., In re Zurko, 111 F.3d 887 (Fed. Cir. 1997).

The Examiner has failed to meet his burden. Leaving aside the fact that Gong et al. limit their disclosure to establishing the NK-92 cell line, the differences between the NK-92 cells and T-ALL cells known at the time of filing Applicant's claimed method were so great that it was very unlikely that one skilled in the art would have found T-ALL cells to be any teaching with respect to NK-92 cells. The necessary nexus between the NK-92 cells taught by Gong et al. and an *in vivo* treatment of a pathology that would have led one skilled in the art to look to the teachings of Santoli et al. is missing. Simply because a cell line is developed or established in the laboratory does not mean that there is an expectation of success for utilizing that cell line in a clinical setting. Even the inventor of the NK-92 cell line did not initially recognize the importance or utility of the NK-92 cells in a clinical setting. Klingemann Decl., ¶ 24c. Additionally, the mere disclosure of NK-92 cells by Gong et al. is simply insufficient to obviate Applicant's claimed method and the Examiner's attempt to overcome the deficiencies of Gong et al. with the teachings of Santoli et al. is unfounded for a number of reasons, as detailed below.

First, any teaching, suggestion, or incentive in the prior art must not only motivate the skilled artisan to combine the teachings or suggestions, but must do so with a reasonable expectation of success. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art. In re Vaeck, 947 F.2d 488; M.P.E.P. § 2143.03. There is simply no such teaching, suggestion, or motivation in Gong et al. to look to Santoli et al., let alone a reasonable expectation of success in combining those

teachings. As set forth in detail above, the NK-92 cells disclosed by Gong et al. are phenotypically and functionally different from the T-ALL cells disclosed by Santoli et al. Because of these significant phenotypic and functional differences, there was simply no reason apparent to one skilled in the art at the time that Applicant's claimed method was filed to look to Santoli et al.'s teaching of T-ALL cells for any teaching with respect to a method of treating a pathology *in vivo* in a mammal by administering NK-92 cells, as is claimed by Applicant. Klingemann Decl., ¶ 29. Because of the significant and distinctive differences between these cell lines, the applicability and necessary requirements to use one of these cell lines as a method of treating *in vivo* is not applicable to the other, or to any other cell line for that matter. Id. Instead, the usefulness and necessary requirements for each would have to be characterized independently. Id. If one skilled in the art would have combined the teachings of Gong et al. and Santoli et al., the skilled artisan most certainly would not have had a reasonable expectation of success. Id., ¶ 30. In fact, the inventor of the NK-92 cell line has noted that application of the teachings of Santoli et al. to the NK-92 cells disclosed in Gong et al. would not have led to successful results because of the unique characteristics and requirements of the NK-92 cells. Id. Even with impermissible hindsight, one could not combine the teachings of Gong et al. and Santoli et al. to end up with Applicant's claimed method of treating a pathology *in vivo* by administering NK-92 cells because Applicant's NK-92 cell line is phenotypically and functionally different from Santoli et al.'s T-ALL cells.

Second, successful results and evidence of discovery further establish the patentability of Applicant's claimed method of treating a pathology *in vivo*. "[O]bjective evidence such as commercial success, failure of others, long-felt need, and unexpected results must be considered

before a conclusion on obviousness is reached." Minnesota Mining & Manufacturing Co. v. Johnson & Johnson Orthopedics, Inc., 976 F.2d 1559, 1573 (Fed. Cir. 1992) (noting the importance of secondary considerations in the obviousness analysis), citing Hybritech Inc. v. Monoclonal Antibodies, Inc., 802, F.2d 1367, 1379-80, 231 USPQ 81, 90 (Fed. Cir. 1986). Recent clinical trial studies demonstrated the "feasibility of large-scale expansion and safety of administering NK-92 cells as allogeneic cellular immunotherapy in advanced cancer patients and serves as a platform for future study of this novel natural killer (NK)-cell based therapy." Cytotherapy 10(6): 625-632, 2008. The methods used were tailored to NK-92 cells, which are very different from the methods tailored to T-ALL cells. Klingemann Decl., ¶ 35.

The Examiner alleges that:

> Santoli et al. teach that lytic human derived cell lines can be used in vivo to treat disease whilst Gong et al. disclose that NK-92 cells are a lytic human derived cell line. In addition, as per the specification, page 2, last paragraph, use of NK cells and LAK cells to treat cancer in vivo was already known in the art. Gong et al. disclose that the NK-92 cell line displays characteristics of NK cells (see abstract), wherein use of NK cells to treat cancer in vivo was already known in the art.

Final Office Action, ¶ 8. The Examiner's conclusions are overly broad and misrepresent the disclosures of Santoli et al., Gong et al., and Applicant. Santoli et al. do not teach that all lytic human derived cell lines can be used *in vivo* to treat disease. Rather, Santoli et al. teach that T-ALL cells can be used in cancer therapy. See Santoli et al., 1:11-13. One skilled in the art would not extend such a limited teaching with respect to one cell line to be a teaching with respect to any other cell line. Klingemann Decl., ¶ 29. As discussed in detail above, there are significant phenotypic and functional differences between NK-92 cells and T-ALL cells, thereby eliminating any reason for one skilled in the art at the time the claimed method was developed to

look to Santoli et al.'s teaching of T-ALL cells to arrive at a method of treating a pathology *in vivo* in a mammal by administering NK-92 cells. Id., ¶ 29.

While the Examiner relies on Applicant's disclosure in the Specification (2:24-26) that "NK cells and LAK [lymphokine activated killer] cells have been used in both *ex vivo* therapy and *in vivo* treatment in patients with advanced cancer" to support his obviousness rejection, the Examiner fails to consider that NK cells and LAK cells are quite different from the claimed NK-92 cells and that Applicant's disclosure actually details the limitations of using NK and LAK cells *ex vivo* and *in vivo*. See '955 Application, 4:4-23. Applicant recognizes that "[t]here thus remains a need for a method of treating a pathology related to cancer or a viral infection with a natural killer cell line that maintains viability and therapeutic effectiveness against a variety of tumor classes." See '955 Application, 4:24-26. The Examiner has failed to recognize or consider that Applicant's claimed method, as set forth in claim 20, meets this need. See '955 Application, 5:4-5. While it was known in the art to use NK and LAK cells to treat a pathology, it was not known to use NK-92 cells for such a purpose until Applicant's claimed method was discovered. Gong et al.'s recognition in the Abstract that the novel NK-92 cell line "displays characteristics of activated NK-cells and could be a valuable tool to study their biology" does not impact the patentability of Applicant's claimed method because, at that time, there was absolutely no recognition that the NK-92 cells could be used *in vivo* as a method of treating, nor was there a motivation to look to Santoli et al. for such a teaching. Klingemann Decl., ¶ 29.

The Examiner goes on to support his rejection pursuant to 35 U.S.C. § 103(a) on the grounds that "in the post KSR Int'l Co. v. Teleflex Inc. universe, motivation per se is not even required in a rejection under 35 U.S.C. § 103." Final Office Action, ¶ 8. Quoting KSR Int'l Co.

v. Teleflex Inc., 550 U.S. m. 2007 WL 1237837 at 13 (2007), the Examiner states "if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond his or her skill." Notably, the Examiner has acknowledged that "the two types of cells differ in phenotype" but has still concluded that "both the cells described by Santoli et al. and NK-92 are lytic human derived cell lines that can lyse various tumor cells." Final Office Action, ¶ 8. This conclusion is inaccurate because Gong et al. do not teach that NK-92 cells are capable of lysing various tumor cells of different origin or type. Klingemann Decl. ¶ 24. Instead, Gong et al. teach that NK-92 cells demonstrated cytotoxicity against two human leukemic cell lines in studies developed to characterize the newly isolated cell line. Id. Further, given that one skilled in the art would appreciate the significant phenotypic and functional differences between NK-92 cells and T-ALL cells, there would not have been any reason apparent to one skilled in the art at the time the claimed method was developed to look to Santoli et al.'s teaching of T-ALL cells to arrive at a method of treating a pathology *in vivo* in a mammal by administering NK-92 cells. Id., ¶¶ 27, 29. What the Examiner fails to appreciate is that Santoli et al. only teach methods applicable to T-ALL cells and do not provide guidance as to any other cell lines, while Gong et al. identify and partially characterize NK-92 cells which, at the time, was a new cell line. As discussed above, the inventor of the NK-92 cell line has noted that these two cell lines are from different cell lineages derived from different disease categories, leukemia and lymphoma. Id., ¶ 28. The T-ALL cell lines were derived from a patient with ALL, whereas the NK-92 cell line was derived from a patient with an aggressive LGL lymphoma. Id. The actual application of a method for treating a pathology *in vivo* in a mammal by administering

NK-92 cells would not have been obvious to a person of ordinary skill in the art based on the methods and teachings disclosed in Santoli et al. Id., ¶¶ 29, 30. The phenotypic and functional differences between the cells inherently prevent the know-how from one to be automatically transferred to the other, especially with any expectation of success. Id. Thus, contrary to the Examiner's conclusion, because Gong et al. do not teach a method of treating a pathology *in vivo*, it could not be obvious to use Gong et al. to arrive at, let alone improve, another technique.

The Examiner also asserts that "there is no teaching in Gong et al. that NK-92 cells are unacceptable for in vivo use." Final Office Action, ¶ 8. That notation, however, is irrelevant. It is the teaching of the reference that is relevant to an obviousness analysis, not what the reference does not teach. See, e.g., M.P.E.P. § 2143.01, citing KSR Int'l v. Teleflex Inc., 127 S.Ct. 1727, 1740-1741 (2007) (stating that "rejections on obviousness cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness"). Gong et al. do not teach or suggest that the NK-92 cells disclosed therein could be used *in vivo* to lyse tumor cells. Klingemann Decl., ¶ 24. This together with the fact that Santoli et al.'s teaching is limited to T-ALL cells renders the Examiner's combination of Gong et al. and Santoli et al. unsubstantiated.

The Examiner cites to M.P.E.P. § 2121, stating that "[w]hen the reference relied on expressly anticipates or makes obvious all of the elements of the claimed invention, the reference is presumed to be operable. Once such a reference is found, the burden is on the applicant to provide facts rebutting the presumption of operability." Final Office Action, ¶ 8. For the reasons set forth above, the Examiner has not established a *prima facie* case of obviousness. Accordingly, the burden has not moved to Applicant to rebut the presumption of operability.

However, even if the burden has moved to Applicant, the combination of Gong et al. and Santoli et al. would not have led to successful results because of the unique characteristics and requirements of these cells. Klingemann Decl., ¶ 30.

The Examiner also states that

> obviousness requires only a reasonable expectation of success. Regarding the Klingemann declaration, Santoli et al. teach that there is a need for cytotoxic cell lines which could be used to treat cancer. In view of the high level of skill in the art (Ph.D. or MD, with extensive research training) it would have been obvious to a routineer that other cytotoxic cell lines could be potentially used as per Santoli et al. In addition, the use of NK cells to treat cancer in vivo was already known in the art whilst Gong et al. disclose that the NK-92 cell line displays characteristics of NK cells.

Final Office Action, ¶ 8. As discussed above, there was not a reasonable expectation of success. As emphasized in the declaration of the inventor of the NK-92 cell line, the significant phenotypic and functional differences between NK-92 cells and T-ALL cells rendered the use of one of these cell lines as a method of treating *in vivo* inapplicable to the other, or to any other cell line for that matter, thereby precluding any expectation of success. Klingemann Decl., ¶¶ 29, 30. Additional comparative studies of NK-92 cells and TALL-104 cells further demonstrate that these cell lines are functionally quite different, with NK-92 cells having significantly higher cytotoxic activity than TALL-104 cells. Id., ¶ 31. For example, many hematological cancers are susceptible to killing by NK-92 cells, whereas these cancers are mostly resistant to lysis by TALL-104 cells. Id. In fact, data disclosed in the '955 Application demonstrate that NK-92 cells are more cytolytic than TALL-104 cells or YT cells. Id., ¶ 32. As further evidence of non-obviousness, the methods developed and being used in the clinic are very different for the two

cell lines. Reliance on the teachings of Santoli et al. would not have led to successful use of the NK-92 cells in a clinical setting. See, e.g., Id., ¶ 35.

With respect to Applicant's arguments that NK-92 cells and T-ALL cells are distinct cell lines, the Examiner alleges that Tam et al. state that "[a]n alternative is to use established cytotoxic NK tumor cell lines, which would give access to large numbers of effector cells. This concept has been proved by Cesano et al. (1997), who showed that an NK-like cell, TALL-104 was effective in treating a variety of malignancies in dogs." Final Office Action, ¶ 8. The Examiner continues: "contrary to the comments in the Klingemann declaration, Tam et al. disclose that TALL-104 is an NK-like cell line which is similar enough to NK cells that findings using TALL-104 cells can be extrapolated to NK cell lines." Final Office Action, ¶ 8. In fact, as set forth in Dr. Klingemann's declaration, Tam et al. actually demonstrate that NK-92 cells are more stable than T-ALL 104 cells. Klingemann Decl., ¶ 28e. Specifically, Tam et al. demonstrate that NK-92 cells and T-ALL cells are phenotypically distinct because Tam et al. showed that NK-92 cells require >500 Gy to suppress proliferation, while others have reported that T-ALL 104 cells require 40 Gy irradiation to suppress proliferation. See Santoli et al., Cancer Res., 56: 3021-3029, July 1996. Additionally, NK-92 cells maintain cytotoxicity and function even after irradiation, while T-ALL cells lose some cytotoxicity when irradiated. Klingemann Decl., ¶ 28e. NK-92 cells do not require supplemental immunosuppression. Id., ¶ 28f. Accordingly, T-ALL cells are immunogenic while NK-92 cells are not. Id.

The Examiner also alleges that "Klingemann et al. (1996) also disclose that NK-92 and TALL-104 cells have similar lytic properties." Final Office Action, ¶ 8. In fact, that is a misrepresentation of Klingemann et al. That reference actually acknowledges that "[a]

comparative study of the cytotoxic activity of the TALL-104 and the NK-92 cells has suggested, however, that NK-92 cells display a higher level of cytotoxicity than TALL-104 cells against leukemic and lymphoma targets and also lyse a broader spectrum of leukemic target cells including primary leukemias derived from patients." Klingemann et al., Biol. Blood Marrow Transplant., 2:68-75, 73 (1996). As set forth in detail above, data actually have demonstrated that NK-92 cells are, in fact, superior to T-ALL cells. See Klingemann Decl., ¶¶ 31-33.

The Examiner alleges that "there is no evidence of record that in vivo treatment with NK-92 cells is superior to in vivo treatment with TALL-104 cells." Final Office Action, ¶ 8. The Examiner is incorrect. See, e.g., Klingemann Decl., ¶¶ 31-33 (stating that "data disclosed in the '955 Application demonstrate that NK-92 cells are more cytolytic than TALL-104 cells or YT cells"). In fact, data disclosed in the '955 Application demonstrate that NK-92 cells are more cytolytic than T-ALL 104 cells or YT cells. See '955 Application, 35:11-36:20; 39:3-22; 44:11-28; Tables 5 and 6, Fig. 9.

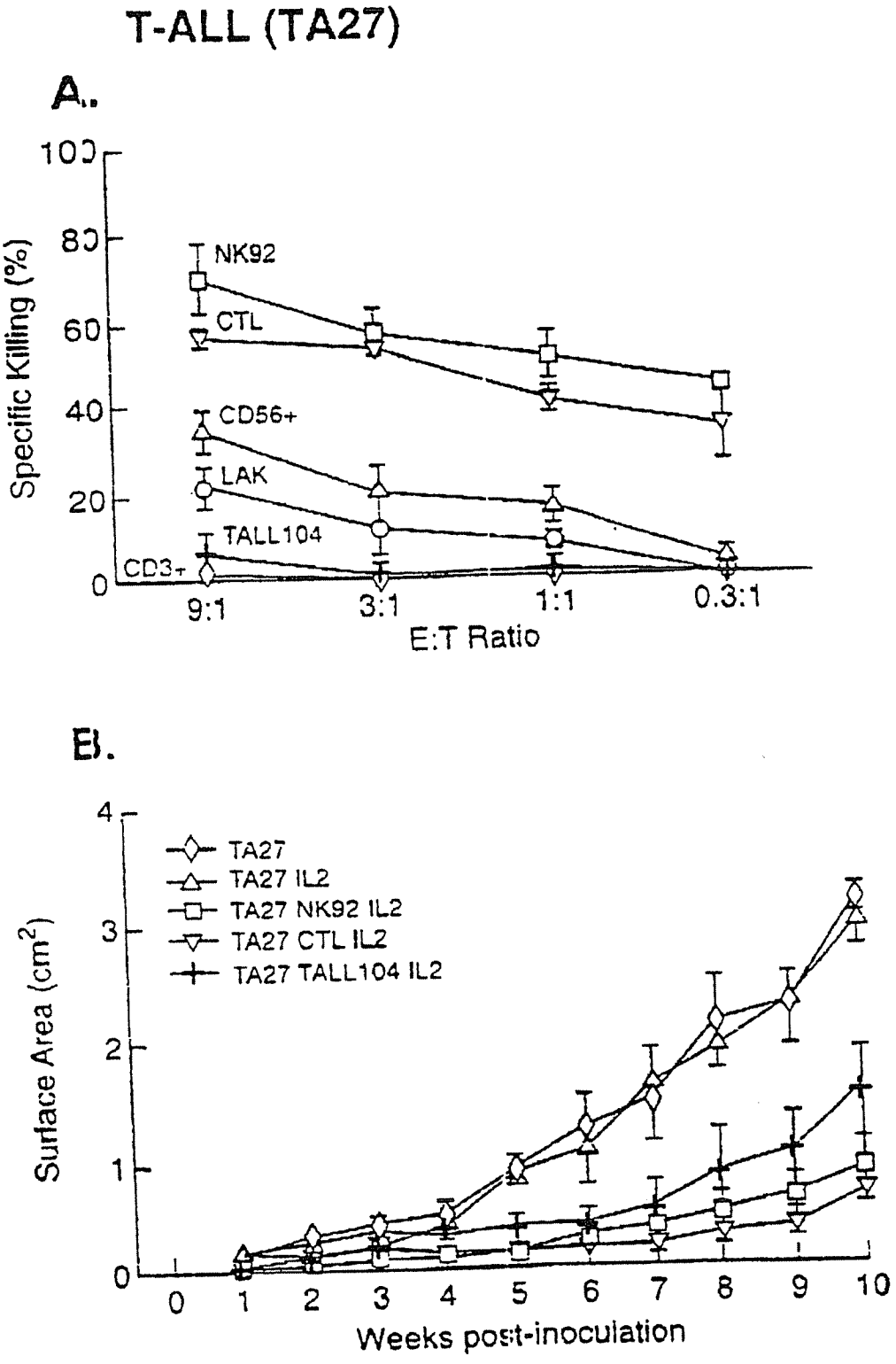
T-ALL (TA27)
A.
100
80
60
40
20
0
Specific Killing (%)
NK92
CTL
CD56+
LAK
TALL104
CD3+
9:1
3:1
1:1
0.3:1
E:T Ratio
B.
4
3
2
1
0
Surface Area (cm2)
TA27
TA27 IL2
TA27 NK92 IL2
TA27 CTL IL2
TA27 TALL104 IL2
0 1 2 3 4 5 6 7 8 9 10
Weeks post-inoculation

FIGURE 9

'955 Application, Fig. 9. These results demonstrate that the NK-92 cell line and the T-ALL 104 cell line are not even comparable. Klingemann Decl., ¶ 32. In fact, the inventor of the NK-92 cell line found these results to be surprising. Id., ¶ 33.

In fact, results recently published by the inventor are promising and encourage continued development of the use of NK-92 cells as a method of treatment. Klingemann Decl., ¶ 35. This study confirmed the feasibility of large-scale expansion and safety of administering *ex vivo* expanded NK-92 cells as allogeneic cellular immunotherapy in patients with refractory renal cell cancer and melanoma. See Arai et al., Cytotherapy, 10(6): 625-632 (2008) (a copy of which is attached hereto).

For at least the reasons discussed above, Gong et al. does not teach or suggest each and every element of Applicant's claimed method of treating a pathology. Because Gong et al. fail to teach or suggest each and every one of Applicant's claimed elements, Santoli et al.'s alleged teaching with respect to *in vivo* treatment by T-ALL cells becomes moot. The addition of Santoli et al. to Gong et al. does not ameliorate the deficiencies of Gong et al. as an obviating reference. Therefore, the rejection of claim 20 cannot stand.

5. Applicant's methods of treating a pathology as set forth in dependent claims 22, 26, 27, and 30 are also patentable over Gong et al. in view of Santoli et al.

The Examiner alleges that dependent claim 22, which depends from claim 20, is also obviated by the combination of Gong et al. in view of Santoli et al. Applicant disagrees with the Examiner's rejection of dependent claim 22 because dependent claim 22 also requires that the pathology is a cancer. Claim 22 is allowable by virtue of its dependency on claim 20, which is allowable for at least the reasons set forth above. *See* M.P.E.P. § 2143.03 (stating that "[i]f an

independent claim is nonobvious under 35 U.S.C. § 103, then any claim depending therefrom is nonobvious"); see also In re Fine, 837 F.2d 1071, 5 USPQ2d 1506 (Fed. Cir. 1988). Accordingly, any teaching with respect to the pathology being a cancer is rendered moot because Gong et al. in view of Santoli et al. fail to teach or suggest each and every element of Applicant's claimed method treating a pathology for at least the reasons set forth above. The Examiner's rejection of claim 22 cannot stand.

The Examiner also alleges that dependent claim 26, which depends from claim 20, is also obviated by the combination of Gong et al. in view of Santoli et al. Applicant disagrees with the Examiner's rejection of dependent claim 26 because dependent claim 26 also requires that the cells be administered to a human intravenously. Claim 26 is allowable by virtue of its dependency on claim 20, which is allowable for at least the reasons set forth above. *See* M.P.E.P. § 2143.03 (stating that "[i]f an independent claim is nonobvious under 35 U.S.C. § 103, then any claim depending therefrom is nonobvious"); see also In re Fine, 837 F.2d 1071, 5 USPQ2d 1506 (Fed. Cir. 1988). Accordingly, any teaching with respect to the route of administration of the cells to the mammal being intravenous and the mammal being human is rendered moot because Gong et al. in view of Santoli et al. fail to teach or suggest each and every element of Applicant's claimed method treating a pathology for at least the reasons set forth above. The Examiner's rejection of claim 26 cannot stand.

The Examiner also alleges that dependent claim 27, which depends from claim 20, is also obviated by the combination of Gong et al. in view of Santoli et al. Applicant disagrees with the Examiner's rejection of dependent claim 27 because dependent claim 27 also comprises the step of administering to the mammal a cytokine that promotes the growth of NK-92 cells. Claim 27

is allowable by virtue of its dependency on claim 20, which is allowable for at least the reasons set forth above. *See* M.P.E.P. § 2143.03 (stating that "[i]f an independent claim is nonobvious under 35 U.S.C. § 103, then any claim depending therefrom is nonobvious"); see also In re Fine, 837 F.2d 1071, 5 USPQ2d 1506 (Fed. Cir. 1988). Santoli et al. do not disclose a method of treating comprising the step of administering to the mammal a cytokine that promotes the growth of NK-92 cells. Rather, Santoli et al. disclose "incorporating into the cell line a selected lymphokine gene." Santoli et al., 7:29-34. Thus, Santoli et al.'s teaching cannot obviate Applicant's dependent claim 27 because the combination of Gong et al. in view of Santoli et al. fail to teach or suggest Applicant's claimed method. The Examiner's rejection of claim 27 cannot stand.

The Examiner also alleges that dependent claim 30, which depends directly from claim 22 (and indirectly from claim 20), is also obviated by the combination of Gong et al. in view of Santoli et al. Applicant disagrees with the Examiner's rejection of dependent claim 30 because dependent claim 30 also requires that the cancer be a solid tumor. Claim 30 is allowable by virtue of its dependency on claim 20, which is allowable for at least the reasons set forth above. *See* M.P.E.P. § 2143.03 (stating that "[i]f an independent claim is nonobvious under 35 U.S.C. § 103, then any claim depending therefrom is nonobvious"); see also In re Fine, 837 F.2d 1071, 5 USPQ2d 1506 (Fed. Cir. 1988). Accordingly, any teaching with respect to the cancer being a solid tumor is rendered moot because Gong et al. in view of Santoli et al. fail to teach or suggest each and every element of Applicant's claimed method treating a pathology for at least the reasons set forth above. The Examiner's rejection of claim 30 cannot stand.

D. Conclusion

For at least the reasons set forth herein, Applicant respectfully requests that the Board reverse the Examiner's final rejection and allow all claims because the Examiner has failed to show or establish how Gong et al. in combination with Santoli et al. obviates Applicant's claimed invention. In accordance with the above remarks, claims 20, 22, 26, 27, and 30 are patentable over the cited references and allowance of same is hereby respectfully requested.

Applicant does not believe that a fee is due. However, if the Commissioner determines that a fee is required, the Commissioner is authorized to charge any required fee to Deposit Account No. 03-2026.

Respectfully submitted,

By: _____

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VIII. CLAIMS APPENDIX

The following claims are the claims on appeal as presently amended:

1. (Withdrawn) A method of purging cells related to a pathology from a biological sample, said method comprising (i) obtaining a biological sample from a mammal, wherein the biological sample is suspected of containing cells related to the pathology, and (ii) contacting the biological sample with a medium comprising NK-92 or modified NK-92 natural killer cells, wherein the modified NK-92 cells have been modified by a physical treatment or by transfection with a vector; whereby the natural killer cells purge cells related to the pathology from the sample.

2. (Withdrawn) The method described in claim 1 wherein the pathology is a cancer.

3. (Withdrawn) The method described in claim 1 wherein the pathology is an infection by a pathogenic virus.

4. (Withdrawn) The method described in claim 3 wherein the pathogenic virus is human immunodeficiency virus, Epstein-Barr virus, crytomegalovirus, or herpes virus.

5. (Withdrawn) The method described in claim 1 wherein the biological sample is human blood or bone marrow.

6. (Withdrawn) The method described in claim 1 wherein the natural killer cell is immobilized on a support.

7. (Withdrawn) The method described in claim 1 wherein the modified NK-92 cells have been modified by a physical treatment that renders them non-proliferative, said treatment not significantly diminishing their cytotoxicity, by treatment that inhibits express of HLA antigens on the NK-92 cell surface, by transfectoin with a vector, or by any combination thereof.

8. (Withdrawn) The method described in claim 7 wherein the cells have been transfected with a vector encoding a cytokine that promotes the growth of the cells, a vector encoding a protein that is responsive to an agent, a vector encoding a cance cell receptor molecule, or with any combination thereof.

9. (Withdrawn) The method described in claim 1 wherein the medium further comprises cytokine that promotes the growth of the cells.

10. (Withdrawn) A method of treating a pathology ex vivo in a mammal comprising the steps of:

(i) obtaining a biological sample from the mammal, wherein the sample is suspected of containing cells related to the pathology;

(ii) contacting the biological sample with a medium comprising NK-92 or modified NK-92 natural killer cells, wherein the modified NK-92 cells have been modified by a physical treatment or by transfection with a vector, whereby the cells related to the pathology in the sample are selectively destroyed, thereby producing a purged sample; and

(iii) returning the purged sample to the mammal.

11. (Withdrawn) The method described in clam 10 wherein the pathology is a cancer.

12. (Withdrawn) The method described in claim 11 wherein the cancer is a leukemia, a lymphoma or a multiple myeloma.

13. (Withdrawn) The method described in claim 10 wherein the pathology is an infection by a pathogenic virus.

14. (Withdrawn) The method described in claim 13 wherein the pathogenic virus is human immunodeficiency virus, Epstein-Barr virus, cytomegalovirus, or herpes virus.

15. (Withdrawn) The method described in claim 10 wherein the biological sample is blood or bone marrow and wherein the mammal is a human.

16. (Withdrawn) The method described in claim 10 wherein the natural killer cell is immobilized on a support.

17. (Withdrawn) The method described in claim 10 wherein the medium comprises modified NK-92 cells which have been modified by a physical treatment that renders them non-proliferative, said treatment not significantly diminishing their cytotoxicity, by treatment that inhibits expression of HLA antigens on the NK-92 cell surface, by transfection with a vector, or by any combination thereof.

18. (Withdrawn) The method described in claim 17 wherein the cells have been transfected with a vector encoding a cytokine that promotes the growth of the cells, a vector encoding a protein that is responsive to an agent, a vector encoding a cancer cell receptor molecule, or with any combination thereof.

19. (Withdrawn) The method of treating a cancer described in claim 10 wherein the medium further comprises a cytokine that promotes the growth of the cells.

20. (Previously presented) A method of treating a pathology *in vivo* in a mammal comprising the step of administering to the mammal a medium comprising NK-92 cells (available from American Type Culture Collection (ATCC) as Deposit No. CRL-2407).

21. (Withdrawn) The method described in claim 20 wherein the modified NK-92 cells have been transfected with a vector encoding a cytokine that promotes the growth of the cells, with a vector encodinga protein that is responsive to an agent, a vector encoding a cancer cell receptor molecule, or with any combination thereof.

22. (Previously presented) The method described in claim 20 wherein the pathology is a cancer.

23. (Withdrawn) The method of treating a pathology described in claim 31 wherein the cancer is a leukemia, a lymphoma or a multiple myeloma.

24. (Withdrawn) The method described in claim 20 wherein the pathology is an infecton by a pathogenic virus.

25. (Withdrawn) The method described in claim 24 wherein the pathogenic virus is human immunodeficiency virus, Epstein-Barr virus, cytomegalovirus, or herpes virus.

26. (Previously presented) The method of treating a pathology described in claim 20 wherein the route of administration of the cells to the mammal is intravenous and the mammal is human.

27. (Previously presented) The method of treating a pathology described in claim 20 further comprising the step of administering to said mammal a cytokine that promotes the growth of said NK-92 cells.

28. (Withdrawn) The method of treating a pathology decribed in claim 26 wherein the NK-92 is modified by transfection with a vector encoding a protein that is responsive to an agent such that when the agent is taken up by the cell, the cell is inactivated, and wherein the method further comprises administering to the mammal, after a time sufficient for the natural killer cell to treat the cancer has elapsed, an amount of the agent effective to inactivate the cell.

29. (Withdrawn) The method of treating a pathology described in claim 28 wherein the agent is acyclovir or gancyclovir.

30. (Previously presented) The method of treating a pathology described in claim 22 wherein the cancer is a solid tumor.

31. (Withdrawn) The method of treating a pathology described in claim 22 wherein the cancer is a non-solid tumor of circulating cells.

IX. EVIDENCE APPENDIX

(1) Declaration of Hans Klingemann, M.D., Ph.D. Pursuant to 37 C.F.R. § 1.132, filed on October 15, 2008, in support of the Request for Continued Examination filed on October 15, 2008, in response to the Final Office Action mailed on April 15, 2008.

(2) Arai et al., Cytotherapy, 10(6): 625-632 (2008), cited in Declaration of Hans Klingemann, M.D., Ph.D. Pursuant to 37 C.F.R. § 1.132, ¶ 35, filed on October 15, 2008, and attached thereto as Exhibit 2.

(3) Tam et al., Hum. Gene Ther., 10:1359-1373, 1999, cited in Declaration of Hans Klingemann, M.D., Ph.D. Pursuant to 37 C.F.R. § 1.132, ¶ 28, filed on October 15, 2008.

(4) Santoli et al., Cancer Res., 56:3021-3029, July 1996, cited in Declaration of Hans Klingemann, M.D., Ph.D. Pursuant to 37 C.F.R. § 1.132, ¶ 28, filed on October 15, 2008.[2]

(5) Cesano et al. (1997), cited in Final Office Action, pg. 8, mailed on March 24, 2009.

[2] This reference was mistakenly cited as Santoli et al. rather than Cesano et al. However, the citation to the journal and page numbers is the same as was cited previously.

Appendix A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
Hans Klingemann	
Serial No. 10/008,955	
Filed: December 7, 2001	**NATURAL KILLER CELL LINES AND METHODS OF USE**
Art Unit: 1644	
Patent Examiner: Ronald B. Schwadron	
Attorney Docket No. 06-129PCT/US/CIP	
Confirmation No.: 5420	

DECLARATION OF HANS KLINGEMANN, M.D., Ph.D.
PURSUANT TO 37 C.F.R. § 1.132

I, Hans Klingemann, M.D., Ph.D., of Boston, Massachusetts, hereby declare that:

1. All statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

2. I am the sole inventor of the modified NK-92 cells disclosed in U.S. Patent Application Serial No. 10/008,955 (hereinafter, "the '955 Application"), identified above.

3. I submit this Declaration in support of the Response To Final Office Action filed on October 15, 2008.

4. I earned my Vor-Diplom in Biology from the University of Heidelberg, Heidelberg, Germany, in 1971, and my M.D. from the University of Wurzburg Medical School, Germany, in 1976. I carried out my internship in Internal Medicine and Surgery at the University of Wurzburg Medical School, Germany, from 1977-1978 and my residency in Internal Medicine at the University of Marburg Medical School, Germany, from 1978-1984. I received additional Post-graduate training in Bone Marrow Transplant/Oncology at the Fred Hutchinson Cancer Research Center, Seattle, WA, from 1984-1986.

5. I have held academic appointments at the University of Marburg Medical School (Privat-Dozent of Medicine, 1983-1986; Professor of Medicine, 1986-1987), University of British Columbia, Vancouver, CDN (Clinical Associate Professor, 1987-1995; Clinical Professor, 1995-1997), RUSH Medical College, Chicago, IL (Coleman Foundation Professor of Medicine, 1997-2004), and TUFTS University School of Medicine, Boston, MA (Professor of Medicine, 2004-present).

6. I have also held hospital/research appointments at the following facilities: Fred Hutchinson Cancer Research Center, Seattle, WA (Research Associate, 1984-1986); University of Marburg Medical School, Germany (Attending Physician, Dept. of Medicine, 1986-1987); Vancouver Hospital and Health Sciences Center, Vancouver CDN (Active Staff, Div. Of Hematology, 1987-1997); British Columbia Cancer Agency, Vancouver CDN (Active Staff, Clinical Hematology, 1987-1997); Vancouver Hospital

and BC Cancer Center, CDN (Attending Physician, Div. Of Hematology, 1987-1997); Leukemia/Bone Marrow Transplant Program of BC (Member, 1987-1997); Terry Fox Laboratory for Hematology/Oncology, BC Cancer Research Center, Vancouver, CDN (Chief, Transplantation Biology Laboratory, 1990-1997); RUSH University Medical Center, Chicago, IL (Director, Section of Bone Marrow Transplant & Cell Therapy, 1997-2004; Medical Director, Sramek Center for Cell Engineering, 2001-2004); TUFTS-New England Medical Center, Boston, MA (Senior Investigator, Molecular Oncology Research Institute, 2005-present; Director, Bone Marrow and Hematopoietic Cell Transplant Program, 2004-present); and TUFTS-NEMC Cancer Center, Boston, MA (Director, Hematologic Malignancy Program, 2007-present).

7. Additionally, I have advised numerous trainees over the course of my academic and professional careers and have taught numerous classes, both at the undergraduate and graduate levels.

8. Over the course of my career, my research projects have included studying various basic and clinical issues in transplantation immunology covering areas such as dendritic vaccines, natural killer cell biology and mesenchymal stem cells. This translational research has resulted in over 150 publications and a variety of innovative clinical trials.

9. I have authored numerous peer-reviewed publications, review papers/editorials, non-peer reviewed publications/conference proceedings, books and book chapters, and abstracts in the fields of translational research, transplantation biology, and tumor

immunology, including a number of publications relating to natural killer cells and NK-92 cells. A list of my publications is attached hereto as Exhibit 1.

10. I have also been invited to make numerous oral presentations to a variety of audiences on topics related to the fields of translational research, transplantation biology, and tumor immunology. A list of my oral presentations is included in Exhibit 1 hereto.

11. I am also a member of the following professional associations:

International Society of Experimental Hematology
American Society of Hematology
International Society for Cell Therapy
American Society for Blood and Bone Marrow Transplantation
American Society for Clinical Oncology.

12. Over the course of my academic and professional careers, I have received numerous awards and honors for my research contributions, including:

Dr. *Med.* (Magna Cum Laude)
Wolf Boas Research Award by the German Society of Gastroenterology for the best Doctoral Thesis
Habilitation (prerequisite for full professorship), University of Wurzburg Medical School, German (Ph.D. equivalent)
German Cancer Research Foundation Fellowship

13. My education, training, laboratory research, teaching experiences, and professional activities have enabled me to develop an expertise in various specialties within the field of translational research, transplantation biology, and tumor immunology, including an expertise on natural killer cells and NK-92 cells, and their use in the treatment of cancers and viruses.

14. Based on my educational background and work experience, I consider myself to be one skilled in the arts of translational research, transplantation biology, and tumor immunology, and particularly in the area of natural killer cells and NK-92 cells.

15. I am the inventor of the modified NK-92 cell line disclosed and claimed in the '955 Application.

16. I have read and am familiar with the '955 Application as it was filed in the U.S. Patent and Trademark Office and the claims of that application as currently pending in the Response To Final Office Action filed herewith.

17. I have reviewed the following prior art references cited by the Examiner of the '955 Application in the Final Office Action mailed on April 15, 2008, and am familiar with the material disclosed therein:

(a) Gong et al., Leukemia, 1994 (hereinafter, "Gong et al."); and

(b) U.S. Patent No. 5,272,082 to Santoli et al. (hereinafter, "Santoli et al.").

18. I am one of the authors of Gong et al. and am the sole inventor of the immortal cell line, NK-92, disclosed therein.

19. I have reviewed the Final Office Action issued for the '955 Application, which was mailed on April 15, 2008 (hereinafter, "Office Action"), and which contains the following statements:

> It would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to have created the claimed invention because Gong et al. teach use of NK-92 cells, while Santoli et al. teach in vivo use of cytotoxic cell lines. One of ordinary skill in the art would have been motivated to do so because Santoli et al. teach that lytic human derived cell lines can be used in vivo to treat disease or in preclinical in vivo studies (see column 10).

Office Action, ¶ 10.

20. The Examiner's statements are incorrect in view of the state of the tumor immunology art at the time that I invented the method of treating a pathology *in vivo* in a mammal by administering NK-92 cells, disclosed and claimed in the '955 Application. One skilled in the art would *not* have combined either Gong et al. with Santoli et al. at that time for at least the reasons set forth in paragraphs 21-40, *infra*.

21. Gong et al. disclosed the NK-92 cell line that I established from peripheral blood mononuclear cells of a fifty-year-old male patient who was diagnosed with an aggressive LGL lymphoma in 1992.

22. At the time that Gong et al. was written, I thought that the NK-92 cell line provided a suitable model to study the biology of NK-cells and activated NK-cells.

23. All experiments disclosed in Gong et al. were performed *in vitro*. Gong et al. partially characterized the cytotoxic profile of NK-92 cells.

24. The Examiner's characterization of Gong et al. is incorrect for at least the following reasons:

a. The Examiner incorrectly states that "Gong et al. teach use of NK-92 cells to lyse leukemic tumor cells." *See* Office Action, ¶ 10. Rather, Gong et al. teach that NK-92 cells demonstrated cytotoxicity against two human leukemic cell lines, but do not teach that NK-92 cells are capable of lysing various tumor cells, including other leukemic tumor cells, of different origin or type.

c. While Gong et al. do not specifically teach that NK-92 cells are unacceptable for *in vivo* use, there is no teaching, suggestion, or motivation in Gong et al. that would lead one skilled in the art to use the NK-92 cell line *in vivo* to lyse tumor cells or as a cancer treatment, much less successfully reduce such a use to practice as a method of treating mammals. In fact, I did not initially recognize the importance or utility of the NK-92 cell line in a clinical setting.

25. Santoli et al. disclose genetically modified cytotoxic T lymphoblastic leukemia cell lines (T-ALL) 104, 107 and 103/2 and their use to treat cancer, both *in vivo* and *ex vivo*. The disclosure in Santoli et al. is limited to T-ALL cells. There is absolutely no teaching or suggestion in Santoli et al. with respect to cell lines in general, or with respect to NK-92 cells in particular, nor is their use described.

26. In fact, I was not aware of Santoli et al.'s T-ALL cell lines at the time that I created the unmodified NK-92 cell line (available from American Type Tissue Collection (ATCC) as Deposit No. CRL-2407) disclosed in Gong et al. or at the time that I arrived at the method of treating a pathology *in vivo* in a mammal by administering NK-92 cells disclosed in the '955 Application.

27. As one skilled in the art, it has been my experience that know-how with respect to one cell line cannot automatically be transferred or applied to another cell line, even where the cells are closely related, including with respect to culture conditions, requirements for growth factors such as IL-2, survival and signaling patterns following adoptive transfer, ability to migrate to tumor sites, sensitivity to chemotherapeutic agents, response to staining with vital dyes, ability to maintain their cytotoxic activity following

radiation, and susceptibility to gene transfer. Furthermore, the know-how required to use a specific cell line as a method of treatment cannot automatically be transferred or applied to another cell line and is dependent on the distinguishing characteristics of each cell line. Simply because one cell line has a specific utility does not mean that other closely related cell lines will have the same utility. Each must be proven independently and the specific conditions necessary for successful results, including treatment, determined.

28. In fact, as set forth below, the T-ALL cell line is not even comparable or related to the NK-92 cell line that I developed and disclosed in Gong et al. Accordingly, there was no reason apparent to one skilled in the art at the time I arrived at the claimed method of treating a pathology *in vivo* in a mammal by administering NK-92 cells to look to Santoli et al.'s teaching of of T-ALL cells for any teaching with respect to methods of treatment with NK-92 cells.

a. The T-ALL cell lines were derived from a patient with ALL, whereas the NK-92 cell line was derived from a patient with an aggressive LGL lymphoma. These two diseases, leukemia and lymphoma, are in different disease categories and the cells derived therefrom are different cell lineages. As such, the cell lines each have unique characteristics in culture and in undergoing proliferation. One skilled in the art would therefore assume that these two cell lines are different and that conclusions with respect to one of the cell lines cannot be drawn to the other cell line.

b. T-ALL cells are of T-cell origin, are CD3-positive (a specific T-cell marker), CD8-positive, rearrange and express the T-cell receptor, are TCRαβ-positive, and are characterized by specific choromosomal translocations. *See* Santoli et al., 1:68, 2:14, and 4:27. In addition, T-ALL cells lack natural cytotoxicity receptors such as NK-44 receptors that are found on NK-92 cells. In contrast, the NK-92 cell line is a true NK cell line (i.e., it is derived specifically from natural killer cells). NK-92 cells are CD3-negative, CD8-negative, do not express or rearrange the T-cell receptor complex (TCR), and have different chromosomal rearrangements than T-ALL cells. As such, one cannot infer the behaviors, transfectability, or cytotoxic mechanisms of NK-92 cells from those of T-ALL cells because the cells have different phenotypes.

c. NK-92 cells have unusual requirements for sub-culturing. Specifically, when cultured *in vitro* in α-minimum essential medium (α-MEM), the American Type Culture Collection (ATCC; Manassas, VA) recommends the media be supplemented with, among other things, 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, 100-200 U/ml recombinant IL-2 (otherwise the cells die after 72 hours), and most surprisingly, a large proportion (25%) of two sera: 12.5% horse serum and 12.5% fetal bovine serum (FBS). In earlier passages, hydrocortisone is necessary. The cell density in culture is critical, and must be regularly checked and regulated by medium changes. The medium formulation, IL-2 concentration, serum concentration and cell density must be carefully regulated throughout the culture period. The culture of these cells is in

contrast to T-ALL cells, which require fetal bovine serum for growth and proliferation, and is similar to other well-established cell lines (or even hybridomas), such as Madin-Darby Canine Kidney (MDCK) cells, which can thrive in simple MEM with 5% (FBS) and 2mM L-glutamine, 10mM N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), and sub-culturing once or twice a week.

d. Santoli et al. teach that T-ALL cells require antibody stimulation with CD2 or CD3 (a specific T cell marker) antigens to express (IFN)-γ, TNF-α, and GM-CSF. *See* Santoli et al. 2:18, 2:47. NK-92 cells do not require antibody stimulation to express (IFN)-γ, TNF-α, and GM-CSF, but rather release these cytokines in response to stimulation by IL-2.

e. Additionally, NK-92 cells are more stable than TALL-104 cells. Tam et al. (Hum. Gene Ther., 10: 1359-1373, 1999) have shown that NK-92 (both wild-type and transfected cells) cells require > 500 Gy to suppress proliferation, while Santoli et al. reported that TALL-104 cells require 40 Gy irradiation to suppress proliferation (*see* Santoli et al., Cancer Res., 56: 3021-3029, July 1996). Additionally, NK-92 cells maintain cytotoxicity and function even after irradiation, while T-ALL cells lose some cytotoxicity when irradiated.

f. Santoli et al. also reported that the standard treatment protocol for clinical trial in dogs required that the dogs be immunosuppressed using CsA, an immunosuppressive drug, starting the day before TALL-104 injections began and continuing through the first two weeks of TALL-104 injections. *See* Santoli et al.,

Cancer Res., 56: 3021-3029, July 1996). NK-92 cells do not require supplemental immunosuppression. These data suggest that TALL-104 cells are immunogenic while NK-92 cells are not.

29. Accordingly, given these significant phenotypic and functional differences between NK-92 cells and T-ALL cells, there was no reason apparent to one skilled in the art at the time I developed the method of treating a pathology *in vivo* in a mammal by administering NK-92 cells to look to Santoli et al.'s teaching of T-ALL cells to arrive at similar method of treatments. Because of the distinctive differences between these cell lines, the applicability and necessary requirements to use one of these cell lines as a method of treating *in vivo* is not applicable to the other, or any other cell line for that matter. The usefulness and necessary requirements for each would have to be characterized independently.

30. For at least the reasons set forth in paragraphs 21-29, *supra*, it would not have been obvious to one skilled in the art at the time the method of treating a pathology *in vivo* in a mammal by administering NK-92 cells was made to have combined the teachings of Gong et al. with Santoli et al. Most certainly one skilled in the art would not have had a reasonable expectation of success. If one skilled in the art were to have applied the teachings of Santoli et al to the NK-92 cells disclosed in Gong et al, they would not have had successful results because of the unique characteristics and requirements of these cells.

31. Additional comparative studies of NK-92 cells and TALL-104 cells further demonstrate that these cell lines are functionally quite different, with NK-92 cells having

significantly higher cytotoxic activity than TALL-104 cells. For example, many hematological cancers are susceptible to killing by NK-92 cells, whereas these cancers are mostly resistant to lysis by TALL-104 cells.

32. In fact, data disclosed in the '955 Application demonstrate that NK-92 cells are more cytolytic than TALL-104 or YT cells. *See* '955 Application, Tables 5 and 6, Fig. 9.

33. Notably, the results demonstrating that the NK-92 cell line is a superior cell line to the TALL-104 cell line were surprising.

34. Given the significant phenotypic and functional differences between NK-92 cells and T-ALL cells and the cytotoxic superiority of NK-92 cells to TALL-104 cells, there was no reason apparent to one skilled in the art as of the filing date of the '955 Application to look to Santoli et al.'s teaching of TALL cells for treatment of disease for any teaching with respect to the NK-92 cells disclosed in Gong et al.

35. Neither of the references cited by the Examiner in the Final Office Action, either alone or in combination, teach or suggest the method of treatment with NK-92 cells disclosed and claimed in the '955 Application and therefore these references do not obviate the claimed method of treating a pathology *in vivo* in a mammal by administering NK-92 cells. In fact, we recently published in Cytotherapy (10(6): 625-632, 2008) Phase I trial results using NK-92 cells based on methods tailored to NK-92 cells, which are very different from methods tailored to TALL cells, and not disclosed or suggested in Santoli et al or Gong et al. *See* Exhibit 2 attached hereto. The results are promising and encourage continued development of the use of NK-92 cells as a method of treatment.

36. Signed at Boston, MA, this 15 day of Oct., 2008.

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EXHIBIT 1

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EXHIBIT 2

NK-92 phase I trial

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Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial

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Background

Renal cell cancer and malignant melanoma are two types of cancer that are responsive to immunotherapy. In this phase I dose-escalation study, the feasibility of large-scale expansion and safety of administering ex vivo*-expanded NK-92 cells as allogeneic cellular immunotherapy in patients with refractory renal cell cancer and melanoma were determined.*

Methods

Twelve patients (aged 31–74 years) were enrolled, three per cohort at cell dose levels of $1 \times 10^8/m^2$, $3 \times 10^8/m^2$, $1 \times 10^9/m^2$ and $3 \times 10^9/m^2$. One treatment course consisted of three infusions. Eleven patients had refractory metastatic renal cell cancer; one patient had refractory metastatic melanoma.

Results

The NK-92 cells were expanded in X-Vivo 10 serum-free media supplemented with 500 U/mL Proleukin recombinant human interleukin-2 (rhIL-2), amino acids and 2.5% human AB plasma. Final yields of approximately 1×10^9 cells/culture bag (218–250 × expansion) over 15–17 days were achievable with ≥ 80% viability. Infusional toxicities of NK-92 were generally mild, with only one grade 3 fever and one grade 4 hypoglycemic episode. All toxicities were transient, resolved and did not require discontinuation of treatment. One patient was alive with disease at 4 years post-NK-92 infusion. The one metastatic melanoma patient had a minor response during the study period. One other patient exhibited a mixed response.

Discussion

This study establishes the feasibility of large-scale expansion and safety of administering NK-92 cells as allogeneic cellular immunotherapy in advanced cancer patients and serves as a platform for future study of this novel natural killer (NK)-cell based therapy.

Keywords

cancer, cell therapy, NK-92, phase I.

Introduction

Treatment options remain very limited for patients with metastatic renal cancer and metastatic melanoma. Median survival is 7–10 months for metastatic renal cancer and metastatic melanoma and both diseases are resistant to chemotherapy and/or radiotherapy [1]. Both cancers, however, seem to be responsive to immunotherapy [2–4] and cellular immunotherapy is increasingly being considered as a form of treatment that is non-cross-reactive with prior chemotherapy and radiation [5,6].

Natural killer (NK) cells are particularly attractive for adoptive cellular immunotherapy because of their unique ability to lyse target cells without priming [7]. Autologous NK cells from cancer patients, however, may be dysfunctional and may not recognize the malignant target. Autologous NK cells may also be inhibited by 'self' HLA expression and some tumors may in fact express functional HLA antigens (Ag) capable of inhibiting NK cell function. Allogeneic NK cells, therefore, potentially represent a better NK cell product for immunotherapy. NK-92 is a human NK-cytotoxic cell line that represents a pure allogeneic activated NK cell source. NK-92 is interleukin-2 (IL-2) dependent, lacks killer cell inhibitory receptors (KIR) and is broadly cytotoxic against a variety of hematologic and solid tumor cell lines, including leukemia, lymphoma, malignant melanoma, prostate cancer and

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breast cancer [8]. *Ex vivo* expansion of NK-92 under good tissue practice (GTP) conditions for clinical use has allowed its entry into phase I study as a novel immunotherapy in advanced cancers [9]. The NK-92 cell line is originally derived from a non-Hodgkin's lymphoma with large granular lymphocyte morphology and a $CD56^+CD3^-CD16^-$ immunophenotype. Studies in SCID mice have confirmed that NK-92 inoculation itself is not leukemogenic. The tumoricidal activity of NK-92 against human leukemias has been tested *in vitro* against leukemic cell lines and primary leukemia cells, as well as *in vivo* by adoptive transfer of NK-92 cells into xenografted SCID mice, with the result of prolonged survival and no signs of leukemia development [10]. NK-92 infusion has further been found to prolong survival in SCID mice inoculated with human malignant melanoma cells, an observation that served as the basis for this clinical trial [11].

The objective of this study was to determine the safety of infusing NK-92 cells in patients with advanced renal cell cancer and melanoma. The three infusions, each given 48 h apart, had no severe side-effects and several patients showed objective anti-tumor responses, suggesting further exploration of this cellular treatment modality in selected cancer indications is warranted.

Methods

Patient eligibility

The study was open from April 2002 to June 2004 at Rush University Medical Center (Chicago, IL, USA). The protocol was approved by the Institutional Review Board and had obtained FDA investigational new drug application status for the *ex vivo* expansion of NK-92 cells. All patients signed informed consent before any study-related procedures. Patients with histologically confirmed metastatic renal cell cancer or malignant melanoma refractory to, or having failed, standard therapy, including surgery, radiation and chemotherapy, were eligible for treatment on this protocol. All patients had measurable disease [by computed tomography (CT) scan or physical examination] and had undergone several prior treatments, including high-dose IL-2 therapy and allogeneic stem cell transplant (SCT). Other eligibility criteria included ECOG 0 or 1, white blood cells (WBC) $>2.0 \times 10^9$/L, Hb >8 g/dl, platelets $\geq 75 \times 10^9$/L, creatinine <2.0 mg/dL and total bilirubin <2.0 mg/dL. Exclusion criteria included ECOG ≥ 2 and concurrent treatment with corticosteroids and/or other immunosuppressive drugs.

Trial design

The trial was a single-center, open-label, dose-escalation study. Three patients were treated at each dose level: 1×10^8 cells/m^2, 3×10^8cells/m^2, 1×10^9 cells/m^2 and 3×10^9cells/m^2. One treatment course consisted of three infusions of the cell dose over 48 h. Infusion days were designated as days 1, 3 and 5. The rationale for the schedule was to infuse as many NK-92 cells before a T-cell directed immune response would theoretically occur.

Manufacturing of the NK-92 cell product

Manufacturing of clinical-grade NK-92 cells was performed under GTP conditions at the Sramek Center for Cell Engineering at Rush University Medical Center [9]. At 3 weeks before the targeted date of infusion, NK-92 cell cultures were initiated from the NK-92 Working Cell Bank. NK-92 cells were expanded in X-Vivo 10 serum-free medium supplemented with 500 U/mL Proleukin recombinant human (rh)IL-2, 0.6 mm l-asparagine, 3 mm l-glutamine, 1.8 mm l-serine and 2.5% human AB plasma. The cultures were initiated at 2.5×10^5 cells/mL in 25 mL (6.25×10^6 cells) in 1-L Vuelife culture bags (American Fluoroseal Corp., Gaithersburg, MD, USA), with the addition of media every 3 days, maintaining a density of 2.5×10^5 cells/mL, and with daily mild disruption of cell aggregates. Final yields of approximately 1×10^9 cells/culture bag (218–250-fold expansion) over 15–17 days was achievable, with $\geq 80\%$ viability. After quality control verification and quality assurance release that included Gram stain, culture and mycoplasma testing, the final NK-92 cell product was resuspended in GM-2 medium (Plasma-Lyte-A medium supplemented with 2.5% human AB plasma) and infused fresh. The last feeding with rhIL-2 and fresh medium was 48 h before the first day of infusion of the expanded NK-92 product. In addition, after completion of the cell culture period, a standard cytotoxicity assay was performed to assess the functional capacity of the *ex-vivo*-expanded NK-92 cells. Calcein AM-labeled K562 and Raji cells were used as targets to determine NK-92 cell cytotoxicity of the *ex vivo*-expanded cells. The NK-92 cells were irradiated with 1000 cGy prior to infusion into the patient (Cesium Source-Blood Bank, Rush University Medical Center).

On the day of infusion, hydration (200 mL NS/h) was given to the patient 2 h prior to the NK-92 cell infusion and continued for 2 h after NK-92 infusion. The total volume of the NK-92 cell product infusate was

100–200 mL, depending on the body weight of the individual patient. The cells were infused at a rate of 5 mL/min, with a total infusion time of approximately 20–30 min. All patients received premedication with diphenhydramine before the start of each cell infusion.

Of note, the NK-92 cell line was being commercialized during the course of the clinical trial.

Treatment and follow-up

Complete tumor staging was performed prior to NK-92 treatment. During cell infusion, patients were closely monitored, with vital signs recorded at 0, 15, 30, 60, 90, 120 and 240 min and every 24 h thereafter. Patients were examined daily for clinical toxicity from NK-92 infusion for the first 7 days and then weekly thereafter until 4 weeks after cell infusion. NCI-CTC version 3 criteria were used to document toxicities. CBC and chemistries were performed daily during the treatment course. CT scans were repeated at 2 and 4 weeks after the treatment course to assess disease response, and thereafter per routine by their local oncologist. Tumor response was assessed according to Response Evaluation Criteria in Solid Tumors (RECIST) [12]. Additionally, a minor response was defined as regression of target tumor lesions by 10–30% with no new lesions and no non-target lesion progression. A mixed response was defined as the regression of some lesions but simultaneous progression of others.

Cytokine assays

Patient sera were collected pre-NK-92 cell infusion (time 0), at 4 h after each infusion on days 1, 3 and 5, and at 7 days post-infusion. The sera at each time point were tested by enzyme-linked immunosorbent assay (ELISA) with a standard multiplexed panel of cytokines (Linco Diagnostic Services Inc., St Charles, MI, USA). The cytokine panel consisted of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, interferon (IFN)-γ, granulocyte–macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF)-α. Four patients had cytokines measured at the higher NK-92 dose level with the hypothesis that the higher cell dose of NK-92 would tend to be more effective.

HLA antibody production

High-resolution DNA typing of the NK-92 cell line was used to establish its HLA type. High-resolution DNA typing for HLA was also performed on two patients for whom 1–2 year follow-up blood samples were available. The patient HLA class I and class II antibody (Ab) production against NK-92 was determined for these samples using standard cytotoxic cross-match and flow cytometric cross-match testing.

Statistical analysis

Analyzes were descriptive and graphical. Under the cytokine analysis, a one-sided sign-test was applied to the data from the four patients who had cytokines measured, to test the significance of the average of pre–post differences.

Results

Patient characteristics

The characteristics of the 12 patients enrolled in the study are summarized in Table 1. The median age was 50 years (range 31–74 years); eight patients were male and four were female. Eleven patients had refractory metastatic renal cell cancer, predominantly clear cell type. One patient had refractory metastatic melanoma, spindle cell type. Prior therapies included nephrectomy, high-dose IL-2, IFN, radiation, chemotherapy and SCT.

Table 1. Baseline characteristics of patients treated with NK-92 ($n = 12$)

Variable	Summary
Median age (years)	50 (range 31–74)
Gender	
Male	8
Female	4
Type of tumor	
Renal cell carcinoma	11
Melanoma	1
Metastatic sites	
Lung	10
Liver	4
Brain/central nervous system	1
Bone	3
Lymph nodes	6
Other	2
Prior therapies	
Surgery	11
IL-2, other immunotherapy (IFN, thalidomide)	10
Chemotherapy	3
Stem cell transplant	1
Radiation	4
Vaccine	1

Toxicity

All 12 patients received the three infusions of NK-92 per protocol and there were no delays in the infusion days. Table 2 summarizes the NK-92-related toxicities during the treatment course. Three patients (patients 8, 9 and 12) experienced grade 1 fevers (range 38.2–38.7°C) during the course of NK-92 infusion and all occurred with the higher dose level of $1 \times 10^9/m^2$. The fevers were self-limited and did not require treatment. The patient with metastatic melanoma developed a temperature of 41°C 4 h after the third infusion of NK-92, which responded to hydrocortisone 100 mg intravenously (i.v.). Blood and urine cultures, as well as culture of the NK-92 bag, were negative. This patient had new onset softening of his bulky pre-auricular and occipital tumor masses with frank drainage from the pre-auricular mass as it softened. There were no serious infections reported for patients at the 1-year follow-up post-NK-92 infusion.

Toxicities that were attributed to the underlying tumor and unrelated to NK-92 infusion included grade 2 neck and chest pains and grade 3 back pain in a patient with bulky retroperitoneal renal cell cancer. One grade 4 hypoglycemic episode (glucose < 20 mg/dL) with symptoms of confusion and seizure-like activity occurred immediately after the first NK-92 infusion in a non-diabetic patient (11) who had extensive liver metastases. The patient's baseline glucose was normal at 162 mg/dL. The hypoglycemia responded to D50 bolus followed by continuous D5 i.v. infusion overnight. No further hypoglycemia episodes occurred with the subsequent two NK-92 infusions.

Clinical outcomes

The follow-up on this study is now 4 years, with all patients followed until death. Patients were allowed to seek other therapies after the 4-week toxicity monitoring period. As a phase I study, the study was not designed to evaluate formally the tumor response or duration of response. One patient (6) had a transient mixed response during the monitoring period. She had extensive metastases in the bilateral lungs, hila, mediastinum, abdominal and retroperitoneal nodes. The mixed response occurred as progression in the mediastinum but reduction in lung masses. She ultimately progressed and died at day 168 post-treatment. Patient 10, with melanoma, had a minor response in a target lesion at the left upper neck that was documented at 2 weeks post-infusion by physical examination and CT scan (Figure 1a,b). This patient, with very advanced disease, subsequently progressed and received alternative therapy, but did survive to 255 days post-NK therapy. Of the 12 patients who completed NK-92 treatment, 11 have subsequently died, 10 from progressive disease. Patient 3, who underwent reduced-intensity allogeneic sibling-matched transplant subsequent to NK-92 treatment, died 2.5 years later from consequences of the post-transplant immunosuppressed state, with bronchopneumonia and no active renal cell cancer. Patient 7 is the only surviving patient post-NK-92 infusion. He had progression at 4 weeks post-NK-92 infusion and went on to receive salvage therapies as allowed by the protocol. He was alive with disease and seeking further therapy for renal

Table 2. Adverse events in patients receiving NK-92 infusions. The severity of adverse events was graded according to NCI-CTC version 3

Subject	Diagnosis	Cell dose/$m^2 \times 3$ doses	Adverse event w/grade (possibly related)
1	RCC	1×10^8	0
2	RCC	1×10^8	0
3	RCC	1×10^8	0
4	RCC	3×10^8	0
5	RCC	3×10^8	0
6	RCC	3×10^8	0
7	RCC	1×10^9	0
8	RCC	1×10^9	1, fever
9	RCC	1×10^9	1, fever
10	Melanoma	3×10^9	3, fever
11	RCC	3×10^9	4, hypoglycemia
12	RCC	3×10^9	1, fever

RCC, renal cell cancer.

Table 3. Clinical outcomes

Subject	Diagnosis	Cell dose/m^2 × 3 doses	Outcome at 4 weeks	Deaths (unrelated to NK-92)
1	RCC	1×10^8	PD*	D1006, PD
2	RCC	1×10^8	PD	D101, PD
3	RCC	1×10^8	PD†	D832, bronchopneumonia
4	RCC	3×10^8	PD	D666, PD
5	RCC	3×10^8	PD	D188, PD
6	RCC	3×10^8	Mixed	D168, PD
7	RCC	1×10^9	PD	Alive D1450
8	RCC	1×10^9	SD	D212, PD
9	RCC	1×10^9	SD†	D1059, PD
10	Melanoma	3×10^9	MR	D255, PD
11	RCC	3×10^9	SD	D695, PD
12	RCC	3×10^9	SD	D466, PD

*RCC, renal cell cancer; PD, progressive disease; SD, stable disease; MR, minor response; D, day. *prior alloSCT; †subsequent alloSCT.*

cell cancer at the latest follow-up, on day 1450 post-NK-92.

Laboratory findings

There was a trend of LDH elevations that occurred with NK-92 infusion at the higher cell dose level of $1 \times 10^9/m^2$ (Figure 2). Patient 8 went from a baseline LDH of 185 U/L to 1269 U/L (normal 200–650 U/L) after the first NK-92 infusion, peaked at 2157 U/L after the third infusion, and remained elevated through day 7 (1493 U/L). Patient 11, with the hypoglycemic episode, had a dramatic increase in her serum LDH to 1219 U/L at 4 h after the first NK-92 infusion. The LDH remained elevated through the subsequent two infusions, 1536 and 1254 U/L, respectively, but normalized at day 14 of the treatment course to 237 U/L. Patient 10, with metastatic melanoma, who developed high-grade fever and a clinical tumor response, similarly had elevation from a baseline normal LDH of 409 U/L to a peak of 791 U/L and 763 U/L on infusion days 3 and 5, respectively, with ultimate normalization to 327 U/L at day 14.

Other laboratory parameters examined did not show clinically significant changes in total WBC, platelets, neutrophil count, lymphocyte count or eosinophil count in patients over the three NK-92 infusions or in the 4 weeks of follow-up.

Cytokines were measured in four of the higher cell dose patients' sera pre-, at 4 h post- each of the three NK-92

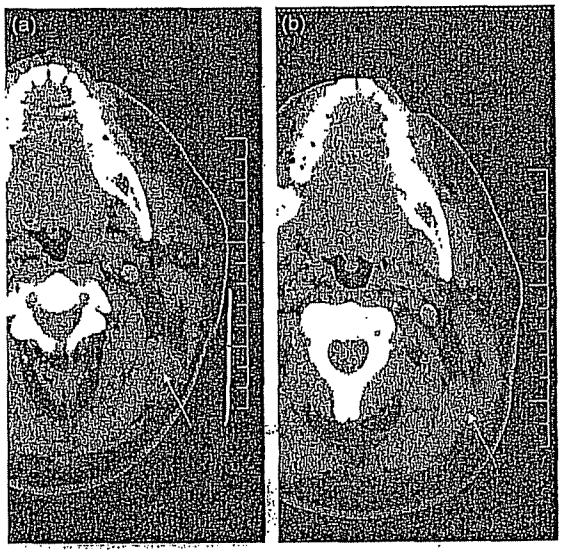

Figure 1. (a) Patient 10, pre-NK-92 infusion, left upper neck mass, 3.15 × 2.54 cm. (b) Two weeks post-NK-92 infusion, shrinkage of left upper neck mass, 2.46 × 1.76cm.

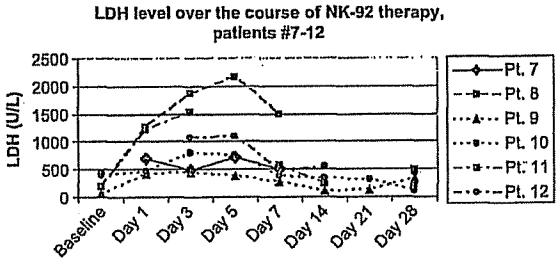

Figure 2. Trend of LDH elevation during NK-92 infusion starting at $1 \times 10^9/m^2$ cell dose. After an initial increase during treatment, the LDH values return to baseline by day 14.

infusions, and at 7 days post-infusion. Positive elevations in IL-6, IL-8 and IL-10 cytokines were seen with NK-92 infusion at the higher cell doses, perhaps suggesting tumor lysis. In patient 10, with metastatic melanoma, clinical tumor shrinkage correlated with a massive rise in IL-6, to 6819 pg/mL from a baseline of 17 pg/mL, along with grade 3 fever. IL-8 and IL-10 similarly rose (Table 4) and then normalized by day 7 post-infusion. Another observation was in patient 9, with metastatic renal cell cancer, who had baseline elevations of IL-4, IL-6 and IL-8, possibly reflecting constitutive cytokine secretion from the renal tumor.

As only four patients had cytokines measured, the sample size limited the degree of statistical reliability. However, if the IL-6, IL-8 and IL-10 pre–post differences (three per patient) are averaged within patients, in all four patients the average pre–post difference was always positive. This has a one-sided sign-test *P*-value of 0.0625, which is the smallest *P*-value obtainable in a non-parametric test with only four patients.

High-resolution HLA typing for NK-92 was confirmed as follows: A3, A11; B7, B44, Bw4$^+$, Bw6$^+$; Cw*07(3R), Cw*1601(3R); DR7, DR15; DQ2, DQ6; DR51$^+$, DR52$^-$, DR53$^+$. Samples from two patients (1 and 11) were tested for the development of anti-HLA Ab against NK-92. Patient 1 was found to have both HLA class I and class II Ab to the NK-92 cell line at 2 years post-exposure. Cytotoxicity and flow cytometric cross-match assays were also positive for this patient. For patient 11, panel reactive Ab and cross-match assays were negative at 1 year post-exposure.

Discussion

The development of the continuously growing NK-92 as a universal donor of highly cytotoxic tumoricidal cells is attractive for allogeneic cellular immunotherapy. Renal cell cancer and melanoma were chosen as the target diseases for this trial based on their previously reported immune responsiveness as tumors [2–4].

The main objective of the phase I trial was to determine the feasibility and safety of administration of NK-92 cell therapy with multiple infusions in these advanced cancer patients. NK-92 cells were successfully expanded under GTP conditions, on average 200–fold over 15–17 days with $\geq 80\%$ viability. Infusional toxicities were generally minimal, limited to grade 1 fevers. No severe hemodynamic or hematologic toxicities were seen with the NK-92 infusion, and thus it compares favorably with other cellular immunotherapies that have used autologous NK or allogeneic haplo-identical NK cells [13–18].

The two major toxicities of grade 3 fever and grade 4 hypoglycemia seen in two patients, while temporally

Table 4. Serum cytokine measurements pre- and post-NK-92 doses. Cytokines were measured in the patients' sera before, 4 post- each of the three NK-92 infusions and at 7 days post-NK-92 infusion. Elevations in IL-6, IL-8 and IL-10 cytokines were seen with NK-92 infusion in the sample of four patients at the higher cell doses, with return to baseline by day 7

Patient	Diagnosis	Cell dose/ $m^2 \times 3$ doses	NK-92 infusion no.	IL-6* (pg/mL) Pre-	Post-	Day 7	IL-8* (pg/mL) Pre-	Post-	Day 7	IL-10* (pg/mL) Pre-	Post-	Day 7
8	RCC	1×10^9	1	34	71		5	15		<3	<3	
			2	215	94		11	6		<3	4	
			3	125	214	35	9	12	10	<3	<3	<3
9	RCC	1×10^9	1	282	307		339	298		41	22	
			2	291	276		257	327		7	74	
			3	284	286	282	299	309	305	7	24	9
10	Melanoma	3×10^9	1	17	18		20	24		<3	<3	
			2	46	29		27	19		66	44	
			3	17	6819	14	20	607	15	<3	159	<3
11	RCC	3×10^9	1	4	13		25	37		42	906	
			2	<3	<3		15	19		32	327	
			3	<3	<3	<3	16	21	31	19	190	96

**The one-sided sign test has a P-value of 0.0625 for the average of pre–post differences.*

related to the NK-92 infusions, could be reflective of tumor lysis responses in these large tumor burden patients versus a reaction to the infusion of cells. The hypoglycemic response in patient 11, who had extensive liver metastases, could be related to tumor-induced hypoglycemia, which has been described in patients with extensive liver metastases [19]. Such a response could be the result of the release of insulin or a humoral hypoglycemic factor, such as an insulin-like substance or diminished glycogen stores in the liver from extensive metastases [19], or ectopic hormone production by the primary renal tumor, such as IGF-2, that can cause hypoglycemia [20]. Hypoglycemia in this setting might also be interpreted as a surrogate for a tumor lysis reaction [21], as may the increase in LDH seen in several patients after infusion of NK-92 . LDH increase is rather non-specific, however, and one cannot rule out other possibilities for the rise in LDH, such as from dead or dying NK-92 cells that were irradiated prior to infusion.

Similarly, elevations in IL-6, IL-8 and IL-10 with NK-92 infusion at the higher cell doses might suggest tumor lysis reaction. However, the cancers themselves can express these cytokines, as can the NK-92 cell line or a toxic response to the infusion of the cells, making it difficult to interpret the cytokine responses in a small sample of patients.

One patient developed HLA Ab whereas another did not. This result may point to a variability in the immune response to NK-92, and this may in part be explained by the variable host immunocompromised status. Other factors to consider are that prior blood product transfusions in the patient could induce an alloimmune response that is cross-reactive with those Ag expressed by NK-92. A larger number of patients will need to be studied to answer this issue. Still, there would seem to be a logical approach in avoiding retreatment of patients having a positive cross-match beyond a 7-day window in order to prevent an anamnestic response.

The exact mechanism of NK-92 killing has not been established; however, it can be hypothesized that NK-92 essentially lacks KIR because of its immature status, and thus target killing is predominantly through its natural cytotoxicity receptors (NKp30 and NKp46) and activating receptor NKG2D [22], rather than a KIR-mediated NK alloreactivity mechanism. The clinical advantage may be that allogeneic NK cellular therapy with NK-92 has a broader spectrum of tumor killing because it overcomes the 'self' MHC molecule restriction, much as has been hypothesized for adoptive transfer of haplo-identical NK cells in patients with cancer [18,23].

Efficacy was not determined in this phase I trial; however, there were two patients with changes in tumor measurement that seemed to meet minor and mixed responses during the study period. These changes were, as expected, transient in this heavily pretreated population. Having determined the safety of infusion and feasibility of large-scale expansion in this initial study, the future plans with NK-92 include a phase II study to determine the biologic activity in other advanced cancers, and to draw on its unique advantage as a cell line to be a platform for genetic engineering to target tumor Ag, such as ErbB2 [24] and CD20 [25], to increase the potential for improved tumor localization and killing efficacy.

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Characterization of Genetically Altered, Interleukin 2-Independent Natural Killer Cell Lines Suitable for Adoptive Cellular Immunotherapy

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ABSTRACT

NK-92 is a highly cytotoxic natural killer (NK) tumor cell line that possesses properties that make it an excellent candidate for adoptive cellular immunotherapy. However, the cytotoxicity of NK cells is dependent on cytokines such as interleukin 2 (IL-2). Although NK-92 cells maintain cytotoxicity for a time after withdrawal of IL-2, clinical use will probably require prolonged treatment with fully activated cells to eliminate disease effectively. The ability to support cytotoxic cells with exogenously administered IL-2 is limited by associated toxicity. Therefore, we describe the transfection of the IL-2-dependent NK-92 cell line with human IL-2 (hIL-2) cDNA by particle-mediated gene transfer to create two IL-2-independent variants, NK-92MI and NK-92 CI, and describe their characterization and comparison with parental cells. Both variants were shown to contain, express, and synthesize the hIL-2 cDNA. IL-2 synthesis was higher in NK-92MI cells compared with NK-92CI cells, with no expression in parental cells. Functionally, the cytotoxicity of all three cell lines was similar and coincubation with IL-2-independent variants did not affect hematopoietic progenitor cells. NK-92MI and NK-92 CI cells were more radiosensitive than NK-92 cells, with proliferation inhibited at lower radiation doses and increased morality and decreased cytotoxicity compared with parental cells. Data presented here show that we have created by particle-mediated gene transfer two IL-2-independent variants of NK-92 that are identical to parental cells in virtually all respects, including high cytotoxic activity. The nonviral transfection of these cells makes them suitable for clinical applications. These IL-2-independent cells should allow prolonged treatment with fully active natural killer cells without the need for exogenous IL-2 support.

OVERVIEW SUMMARY

The ability of the natural killer cell line NK-92 to kill efficiently a broad range of malignant cells makes it suitable for adoptive cellular immunotherapy of cancer. However, these cells are dependent on IL-2 for cytotoxicity as well as viability and proliferation, which could limit their effectiveness if prolonged treatment is needed to effectively eliminate the malignant cells. The present work describes the creation of two IL-2-independent NK-92 variants by particle-mediated gene transfer of the hIL-2 cDNA. It also characterizes and compares the two IL-2-independent NK cell lines with the parental cells. Although the transfected cells are capable of prolonged growth without exogenous IL-2, they are, functionally and phenotypically, virtually identical to the parental cells. They maintain their high cytotoxicity but do not affect normal hematopoietic progenitor cell function. The data presented here suggest that the nonvirally transduced IL-2-independent NK-92 cells may be excellent candidates as agents for adoptive human cellular immunotherapy.

INTRODUCTION

THERE HAS BEEN INTEREST in the use of autologous cytotoxic cells in the treatment of various malignancies. Several studies have reported the use of *ex vivo*-expanded autologous cy-

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totoxic cells, including natural killer (NK) cells, lymphokine-activated killer (LAK) cells, and tumor-infiltrating lymphocytes (TILs) for adoptive cellular immunotherapy of a number of cancers. However, the isolation and large-scale expansion of cytotoxic cells have proven to be technically difficult. As an alternative, some groups have proposed to use cytotoxic cell lines as the immune effector cells (Cesano *et al.*, 1996; Klingemann and Miyagawa, 1996; Klingemann *et al.*, 1996; Lu and Negrin, 1997).

The NK-92 cell line is a natural killer tumor cell line that has been described by our laboratory (Gong *et al.*, 1994). It has a $CD2^+$, $CD3^-$, $CD4^-$, $CD8^-$, $CD56^+$ phenotype that is characteristic of an activated killer cell although it lacks the CD16 Fc receptor. It is a highly cytotoxic cell line with a broad target range. NK-92 cells have been tested against cell lines derived from a wide variety of cancers including acute and chronic lymphoblastic and myelogenous leukemia, lymphoma, myeloma, melanoma, neuroblastoma, and breast and prostate cancer (Gong *et al.*, 1994; Klingemann *et al.*, 1996; Yan *et al.*, 1998; Tam *et al.*, 1999). Furthermore, NK-92 has also been found to be effective against freshly isolated malignant cells, specifically against cells derived from hematologic malignancies such as B lymphocyte-derived acute lymphocytic leukemia (B-ALL), T lymphocyte-derived acute lymphocytic leukemia (T-ALL), and acute myelogeneous leukemia (AML; Yan *et al.*, 1998). These cells have also been found to be effective *in vivo* in severely compromised immunodeficient (SCID) mouse models against such human malignancies as AML (Yan *et al.*, 1998) and malignant melanoma (Tam *et al.*, 1999). These data suggest that NK-92 may be effective as an adoptive cellular immunotherapy.

One factor that must be considered when evaluating the use of NK cells as an immunotherapeutic agent is their dependence on interleukin 2 (IL-2). Cytotoxic activity of both primary NK cells and NK cell lines is strongly correlated with levels of cytokines such as IL-2. Similarly, NK-92 is completely dependent on IL-2 for both viability and cytotoxicity. Although NK-92 does maintain a large portion of its activity following removal of IL-2, the cytotoxicity begins to diminish after 24 hr and declines to about 10% after 3 days (Gong *et al.*, 1994). It is possible, and even likely, that prolonged treatment with these cells may be necessary if these cells are to be used clinically. Although high levels of killing are seen in 4 hr *in vitro* cytotoxicity assays, it is unclear how well these data reflect the ability of NK-92 cells to eliminate minimal residual disease *in vivo* or to purge malignant cells *ex vivo*. It is possible that clinical *in vivo* and *ex vivo* treatment may require prolonged exposure to optimally activated NK-92 cells in order to achieve adequate levels of killing of malignant cells.

Some previously published studies have described attempts to support cytotoxic cell therapy with administration of IL-2. However, this strategy has been limited by the toxicity associated with a high-dose IL-2 treatment regimen. Therefore, the creation of an IL-2-independent form of the NK-92 cell line offers several advantages. First and foremost, this would allow the IL-2-independent strain to maintain a high level of cytotoxicity over a prolonged period of time and not be constrained by its ability to kill in the absence of IL-2. From a clinical viewpoint, this would allow the possibility of prolonged treatment with optimally active NK-92 cells without the need for exogenous IL-2. Second, the maintenance and expansion of an IL-2-independent NK-92 variant would be much simpler relative to the parental line, alleviating the need to supplement the medium with IL-2. This would be particularly important for large-scale clinical expansion of the NK cells. In this work, we describe the nonviral transfection of NK-92 cells with the cDNA for human IL-2 (hIL-2) by particle-mediated cell transfer to create two IL-2-independent NK cell lines, NK-92MI and NK-92CI. Furthermore, we characterize the IL-2-independent strains and compare them to the parental strain to assess their potential use as an immunotherapeutic agent for the treatment of human malignancy.

MATERIALS AND METHODS

Cell lines

The parental NK-92 cell line was cultured in alpha medium supplemented with 2 m*M* L-glutamine, 0.2 m*M* *i*-inositol, 20 m*M* folic acid, 10^{-4} *M* 2-mercaptoethanol, 12.5% fetal calf serum (FCS), and 12.5% horse serum (Myelocult; Stem Cell Technologies, Vancouver, BC, Canada) in the presence of 100–150 units (U) of human IL-2 (Amgen [Thousand Oaks, CA] or Biotest Pharma GmbH [Dreieich, Germany]). The erythroleukemic cell line K562, the lymphoblastic cell line Raji, and the lymphoma cell line Daudi were all obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1610 supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT). All cell culture media and solutions (RPMI 1610, Dulbecco's phosphate-buffered saline [PBS], Hanks' balanced salt solution, trypsin, etc.) were obtained from Stem Cell Technologies.

DNA clones

The MFG-hIL2 vector (Fig. 1, top) was generously provided by C. Jordan (formerly of Somatix, Alameda, CA). The pCEP4-LTR-hIL2 vector (Fig. 1, bottom) was created by excising the *Hin*dIII–*Bam*HI fragment from the MFG-hIL2 vector, containing the 5′ long terminal repeat (LTR) and hIL-2 gene, and inserting it into the complementary sites of the pCEP4 episomal vector (InVitrogen, Carlsbad, CA). cDNA for human tumor necrosis factor α (TNF-α) was a generous gift from K. Humphries and the cDNA for lymphotactin was isolated by reverse transcription-polymerase chain reaction (RT-PCR) of RNA isolated from human NK cells by B. Henneman (both from the Terry Fox Laboratory, Vancouver, BC, Canada).

Particle-mediated gene transfer

NK cells were transduced by particle-mediated gene transfer using the Biolistic PDS-1000/He particle delivery system (Bio-Rad Laboratories, Hercules, CA). Cells were transduced according to manufacturer instructions. Briefly, 1.0- or 1.6-μm gold particles were disaggregated by sonication and coated with 5 μg of DNA using calcium chloride, spermidine, and ethanol. Thirty-five-millimeter tissue culture plates were coated with poly-L-lysine (Sigma, St. Louis, MO) by incubation overnight at 4°C in sodium bicarbonate buffer (pH 9.5). NK-92 cells, in 1× PBS, were placed into the coated dishes and allowed to ad-

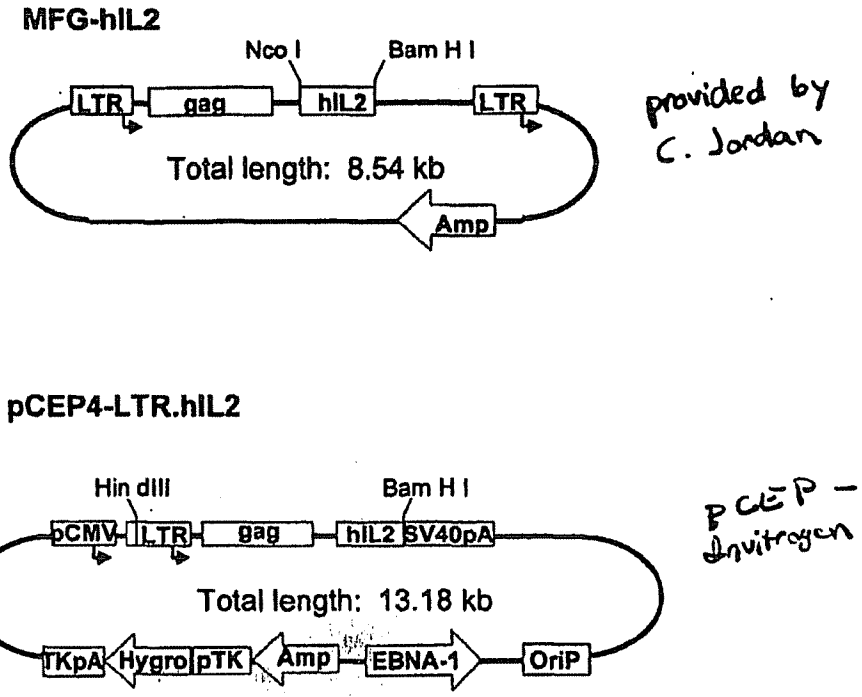


FIG. 1. Human IL-2 vectors. NK-92 cells were transfected with the MFG-hIL2 and pCEP4-LTR-hIL2 vectors. The MFG-hIL2 was provided by C. Jordan (formerly of Somatix). The pCEP4-LTR-hIL2 vector was constructed by ligating a *Hin*dIII–*Bam*HI fragment from the MFG-hIL2 vector containing the 5' LTR, *gag*, and hIL-2 genes into compatible sites in the multiple cloning site of the pCEP4 vector (InVitrogen). Vectors were transferred into the NK-92 cells by particle-mediated gene transfer with the Bio-Rad Biolistic PDS-1000/He system.

here for 30–60 min. PBS was gently aspirated and cells were bombarded two to four times in an evacuated chamber (vacuum of 500mm Hg mercury) with DNA-coated particles accelerated by a 1100-psi helium pulse. Cells were returned to IL-2-supplemented Myelocult medium immediately following bombardment and allowed to recover for 24 hr prior to transfer to IL-2-free medium. Medium was changed periodically and monitored for IL-2-independent growth.

Flowcytometric analysis

NK cells capable of IL-2-independent growth were phenotypically analyzed by either indirect immunofluorescence with unlabeled primary murine anti-human antibodies and fluorescein (FITC)-conjugated goat anti-mouse $F(ab')_2$ immunoglobulin (Ig) or by direct immunofluorescence analysis using murine monoclonal antibodies directly conjugated with FITC or phycoerythrin (PE). Live cells (gated on propidium iodide; Sigma) were analyzed for surface antigen expression using a Becton Dickinson (San Jose, CA) FACScan or FACSort flow cytometer. Cells were preincubated in 10% pooled human serum to avoid nonspecific binding prior to incubation. NK cells were incubated with FITC- or PE-conjugated antibodies against CD2, CD3, CD4, CD8, CD16, and CD56 (Becton Dickinson, Toronto, ON, Canada). Anti-CD25 (human IL-2 receptor, α chain) antibodies were obtained from Becton Dickinson (clone 2A3) or Beckman-Coulter (Miami, FL; clone B1.49.9). Anti-IL-2 receptor β-chain antibodies were provided by either M. Tsudo through J. Hakimi (Hoffmann-La Roche, Nutley, NJ; clone MIK-beta-1) or Beckman-Coulter (clone CFI). Anti-CD28 antibody (clone 9.3) was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, WA). The anti-LFA-1 antibody (CD11a, clone NB-107) was provided by F. Takei of the Terry Fox Laboratory. Anti-CD54 (ICAM-1, clone RR1/1) was provided by R. Rothlein (Boehringer-Ingelheim, Ridgefield, CT), anti-CD102 (ICAM-2, clone CBR IC2/2) was provided by T. Springer (Boston, MA), and anti-CD50 (ICAM-3, clone ICR-2) was provided by M. Gallatin (ICOS Corporation, Bothell, WA).

PCR and Southern blot analysis

The transfection of the NK-92 cells was confirmed by PCR analysis of DNA isolated from both the parental and transfected NK-92 cell lines for the presence of genomic and cDNA forms of the human IL-2 gene. DNA was isolated using DNAzol (GIBCO-Life Technologies, Burlington, ON, Canada). Briefly, cells were lysed in DNAzol and DNA was precipitated with ethanol at room temperature. DNA pellets were collected, washed in 95% ethanol, and briefly air dried. DNA was resuspended in 8 m*M* sodium hydroxide at 62°C and the solution was neutralized with HEPES buffer. DNA was quantitated by

ultraviolet (UV) absorbance at a wavelength of 260 nm. Primers flanking intron 1 of the human IL-2 gene (forward, 5'-CAA CTC CTG TCT TGC ATT GC-3'; reverse, 5'-GCA TCC TGG TGA GTT TGG G-3'; GIBCO-Life Technologies) were used to amplify the DNA (30 cycles, 1 min of denaturation at 95°C, 2 min of annealing at 50°C, and 2 min of elongation at 72°C). PCR products were resolved on a 2% agarose gel. For Southern blot analysis, DNA was transferred to Hybond+ nylon membrane (Amersham-Life Sciences, Arlington Heights, IL) by capillary transfer in 10× SSC (1.5 *M* sodium chloride, 1.5 *M* sodium citrate) and fixed by UV cross-linking (StrataLinker; Stratagene, La Jolla, CA). The blot was hybridized with a ^{32}P-radiolabeled human IL-2 probe for 8–12 hr, washed, and visualized by autoradiography at −70°C with X-Omat XAR film (Eastman Kodak, Rochester, NY).

Northern blot analysis and RT-PCR

Cytokine and chemokine gene expression was analyzed by Northern blot analysis and RT-PCR. RNA was extracted from parental and transfected NK-92 cell lines using Trizol reagent (GIBCO-Life Technologies) according to manufacturer instructions. Briefly, cells were lysed in Trizol and the lysate extracted with chloroform. The aqueous phase was then precipitated with isopropanol. The RNA pellet was collected, briefly air dried, and then resuspended in diethyl-pyrocarbonate-treated water (Sigma). RNA was quantitiated by determining the optical density at 260 nm ($OD_{260\ nm}$). For Northern blot analysis, 15 μg of RNA was resolved on a 1% formaldehyde agarose gel in 1× MOPS [3(*N*-morpholino)propanesulfonic acid; Sigma] and blotted as described previously for Southern blot analysis. The blot was hybridized with ^{32}P-radiolabeled probes for human IL-2, TNF-α, lymphotactin, and β-actin.

Lymphotactin is encoded by two closely related genes denoted SCM1α and SCM1β. Since Northern blot analysis is unable to distinguish between the two genes, expression of these genes was analyzed by RT-PCR (Yoshida *et al.*, 1996). RNA (2.5 μg) was reverse transcribed using a lymphotactin-specific primer (5'-TAATTTTATTCATGCAGTGCTTTCATA-3'). The cDNA was PCR amplified for 35 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 2 min) with primers complementary to both SCM1α and SCM1β (forward primer, 5'-TCAGCCATGAGACTTCTC-3'; reverse primer, primer used for reverse transcription reaction) to give rise to 456-bp fragments from both genes. To distinguish between SCM1α and SCM1β products, the PCR product was digested with the restriction enzyme *Pma*CI (Boehringer Mannheim, Indianapolis, IN), which cuts the SCM1β PCR product into two fragments of 226 and 230 bp while leaving the SCM1α product intact. Ten- and 1-μg aliquots were resolved on a 2% agarose gel and analyzed by Southern blot analysis as previously described.

DNA probes for Northern and Southern blot analysis were radiolabeled by random primer extension. DNA probes for human IL-2, TNF-α, lymphotactin, and β-actin were purified by digestion of the vectors with appropriate restriction endonucleases to excise the cDNA insert and separation by agarose electrophoresis. The DNA fragment was excised from the gel and purified by centrifugation through a Spin-X tube filter (Corning-Costar, Cambridge, MA), phenol–chloroform extraction, and ethanol precipitation. DNA probe was labeled with [α-^{32}P]-dCTP (specific activity, 3000 Ci/mmol; ICN, Montreal, PQ, Canada).

Cytokine determination

IL-2 and TNF-α production by NK-92 cell lines was determined by enzyme-linked immunosorbent assay (ELISA). One million-cell aliquots of the parental or transfected NK-92 cells were cultured in 8 ml of IL-2-free Myelocult medium for 1, 2, and 3 days. Supernatents were collected and frozen at −20°C until all samples were collected. Samples were thawed and assayed for cytokine levels by ELISA according to manufacturer instructions (Quantikine; R&D Systems, Minneapolis, MN). The IL-2 (horseradish peroxidase/tetramethylbenzidine-based colorimetric assay) and TNF-α (high-sensitivity assay; alkaline phosphatase/alcohol dehydrogenase/iodonitrotetrazolium violet-based colorimetric assay) ELISA microtiter plates were read at 450 nm (with a 540-nm correction) and 490 nm (with a 650-nm correction), respectively, in a microplate reader (model EL309 [Bio-Tek Instruments, Winooski, VT] or SpectraMax250 [Molecular Dynamics, Sunnyvale, CA]).

Cell proliferation assay

To analyze the proliferation of the parental and transfected cell lines, cells were plated in triplicate at a concentration of 5×10^4 cells/ml in six-well tissue culture plates (Becton Dickinson). NK-92 cells were plated in IL-2-supplemented medium while NK-92MI and NK-92CI cells were plated in IL-2-free medium. Cells were enumerated by trypan blue exclusion to monitor growth over 7 days. As well, cells were subcultured at day 3 under the conditions described and growth was monitored over 5 days.

Hematopoietic progenitor cell assay

To determine if transected NK-92 cells influenced the clonogenic potential of normal hematopoietic cells, a hematopoietic progenitor cell assay was performed according to established procedures (Cashman *et al.*, 1990). Briefly, 6×10^5 normal peripheral blood mononuclear cells (PBMCs) were cocultured with varying numbers of irradiated NK-92 transfectants (1000 cGy) at ratios ranging from 1000:1 to 1:1 (PBMCs to NK-92 cells) for 2 days. Two hundred thousand cells were then plated in replicate aliquots of methycellulose-containing medium (Stem Cell Technologies) at dilutions to give approximately 10–100 colonies of either erythroid cells (burst forming units-erythroid [BFU-E] cells), granulocytes and macrophages (colony-forming units-granulocyte/macrophage [CFU-GM] cells), or a combination (colony-forming units-granulocyte/erythroid/macrophage/megakaryocyte [CFU-GEMM] cells). Cells were counted under an inverted microscope after 14 days.

Cell-mediated cytotoxicity assay

Cell-mediated cytotoxicity was determined by a ^{51}Cr release assay as previously described (Klingemann and Wong, 1996). Briefly, 5×10^5 cells of each target cell line (K562 and Raji) were labeled with 50 μCi of Na_2CO_4 (NEN-Life Sciences, Mississauga, ON, Canada) for 1 hr at 37°C. Ten thousand target cells were then placed into round-bottomed 96-well plates (Nunc-GIBCO-Life Technologies, Burlington, ON, Canada)

and NK-92 cells were added to target cells at target-to-effector ratios of 1:1, 1:5, 1:10, and 1:20. Cells were incubated for 4 hr and 100 μl of supernatent was removed from each well. The amount of ^{51}Cr released was measured in a γ counter (Beckman Instruments, Mississauga, ON, Canada) and specific cytotoxicity was determined as follows:

Percent cytotoxicity =

$$\left(\frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}}\right) \times 100$$

Radiation of NK-92 cells

To determine the sensitivity of both parental and transfected NK-92 cells to radiation, cells were irradiated using a Cis Bioln-ternational 437c cesium source (Cis-US, Bedford, MA). Cells were collected, washed, and resuspended in medium and irradiated in 15- or 50-ml conical centrifuge tubes (Becton Dickinson). After radiation, cells were washed and resuspended in Myelocult with (for parental NK-92) or without (for transfected cells) IL-2. Cells were cultured for 24, 48, and 72 hr and assayed for viability and proliferation by trypan blue exclusion and [^{3}H]thymidine incorporation and cytotoxicity by ^{51}Cr release assay (as described above). Proliferation was quantitated using a standard thymidine incorporation assay (Klingemann *et al.*, 1994). Briefly, cells were plated in Myelocult (with or without IL-2) in 96-well flat-bottom plates at 4×10^4 cells/200 μl per well. Cells were incubated for 72 hr at 37°C before medium was changed to thymidine-free RPMI for 2 hr. Half a microcurie of [^{3}H]thymidine (ICN) was added to each well and cells were incubated for 4 hr prior to harvesting. Tritium uptake was measured in a liquid scintillation counter.

RESULTS

Transfection of NK-92 cells

Parental NK-92 cells were transfected with either the MFG-hIL2 or pCEP4-LTR-hIL2 vector by particle-mediated gene transfer. From preliminary experiments to establish gene transfer conditions, transfer efficiencies of 5–15% were seen with this methodology (Y. Tam, unpublished observations, 1998). NK cells transfected with the vectors containing the cDNA for human IL-2 were cultured in IL-2-free medium to select for cells that had taken up and were expressing the gene. For NK cells transfected with the MFG-hIL2 vector, 85–95% of cells died 4–7 days after transfer to unsupplemented medium. A small number of cells remained viable, and were assumed to be cells that had been successfully transfected. Virtually all of these cells also began to die and no viable cells were detectable after 2 to 3 weeks. This was expected as the MFG-hIL2 vector construct does not contain the genetic elements required for replication and maintenance in eukaryotic cells, such as a mammalian origin of replication. Therefore, as the transfected cells were maintained in culture and began to replicate, the vector construct would have been lost and the cells would have reverted to their IL-2-dependent phenotype. These cultures were maintained for several weeks although no viable cells were observed by examination under an invested phase-contrast microscope. Surprisingly, a small number of viable cells appeared in the cultures approximately 3–4 weeks after initial transfer of the cells to IL-2-free medium. These cells were capable of IL-2-independent growth on subculturing to fresh medium and appeared to be stably transfected, maintaining their IL-2-independent phenotype during prolonged culturing and passaging. Since the vector was unable to replicate, the appearance of stably transfected cells indicated that the vector had most likely integrated into the genome of a transfected cell. Since this would be a rare event, these transfected cells probably arose from a small number of cells, possibly even a single cell. The kinetics of the development of these transfected cells would be consistent with this hypothesis. IL-2-independent NK-92 cells arising from transfection with the MFG-hIL2 vector were denoted as NK-92MI.

Initial observations for cells transfected with the episomal vector pCEP4-LTR-hIL2 were identical to those seen with NK-92MI. The majority of NK-92 cells died within 4–7 days after transfer to IL-2-free Myelocult medium. However, unlike the NK-92MI cells, the remainder of the cells, which had been transfected, did not lose their IL-2-independent phenotype and die after the initial 2- to 3-week period. Instead, the cells that were initially IL-2 independent were immediately capable of long-term IL-2-independent growth and survival. This was expected as the pCEP4-LTR-hIL2 vector contains elements that enable it to replicate and be maintained in eukaryotic cells as an autonomously replicating genetic element. Therefore, any cell that was initially transfected would be able to maintain its IL-2-independent phenotype for an indefinite length of time. Although cells harboring episomal vectors are not stably transfected by strict definition, these cells were under constant selection pressure, as only cells that maintained the vector would be capable of growth in IL-2-free medium. Therefore, these cells were capable of long-term growth in IL-2-free medium. IL-2-independent NK-92 cells arising from transfection with the pCEP4-LTR-hIL2 were denoted as NK-92CI.

To confirm that NK-92MI and NK-92CI had in fact been transfected with the hIL-2 gene, PCR analysis was performed on the parental and transfected cell lines to confirm that both NK-92MI and NK-92CI contained the cDNA form of the hIL-2 gene. Primers flanking the 88-base pair (bp) intron 1 of the hIL-2 gene were used to amplify DNA isolated from NK-92, NK-92MI, and NK-92CI to assay for the presence of the genomic and cDNA forms. As expected, agarose gel electrophoresis of the PCR products from the parental line revealed a single 263-bp fragment corresponding to the DNA fragment amplified from the genomic IL-2 gene. However, analysis of both the NK-92MI and NK-92CI products revealed two bands, the 263-bp fragment corresponding to the genomic hIL-2 gene as well as a 175-bp fragment resulting from the amplification of the hIL-2 cDNA. To confirm the identity of these DNA fragments, Southern blot analysis with a radiolabeled probe specific for hIL-2 probe was performed. As seen in Fig. 2, both the 263-bp genomic fragment and the 175-bp cDNA fragment hybridized with the probe. These data indicate that both NK-92MI and NK-92CI were successfully transfected and contained the cDNA for hIL-2. Transfection of NK-92MI was confirmed by genomic Southern blot analysis (data not shown), which revealed an additional IL-2 band in NK-92MI. This also confirmed that NK-92MI was a clonal population derived from a single cell that underwent a single integration event. Repeated

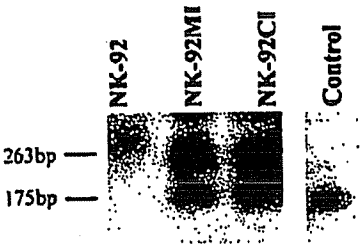


FIG. 2. PCR analysis of NK-92, NK-92MI, and NK-92CI for human IL-2 cDNA. DNA isolated from the parental NK-92 cells and from the NK-92MI and NK-92CI transfectants was subjected to PCR analysis with primers flanking the first exon of the human IL-2 gene. PCR products were resolved on a 2% agarose gel, stained with ethidium bromide, and viewed on a UV transilluminator. DNA was transferred to a nylon membrane and analyzed by Southern blot analysis with a radiolabeled probe for hIL-2. Analysis of the PCR products indicated that NK-92 had only the genomic form (263-bp fragment) of the hIL-2 gene, whereas NK-92MI and NK-92CI possessed both the genomic form and the cDNA form (263- and 175-bp fragments, respectively).

analysis of IL-2 cDNA in NK-92, NK-92MI, and NK-92CI cells during prolonged culture over several months indicated that both the integrated hIL-2 and the pCEP4-LTR-hIL2 vector were stably maintained in NK-92MI and NK-92CI, respectively.

Analysis of gene expression

After successfully transfecting the NK-92 cell line and creating two IL-2-independent cell lines, NK-92MI and NK-92CI were characterized and compared with the parental cells. To analyze expression of specific cytokines and chemokines in the parental and transfected cell lines, they were analyzed by Northern blot analysis. RNA isolated from the NK-92, NK-92MI, and NK-92CI cells was separated by electrophoresis, transferred to nylon membrane, and hybridized with probes for the cytokines hIL-2 and hTNF-α as well as a probe for the chemokine lymphotactin.

Northern blot analysis of IL-2 in these cells revealed that IL-2 RNA was not detectable in the parental cell line (Fig. 3A). However, hIL-2 RNA was found in RNA from both NK-92MI and NK-92CI. Two mRNA transcripts were seen in NK-92MI, a minor transcript at 2.4 kb and a major RNA species of approximately 1.9 kb. In NK-92CI a faint hIL-2 mRNA transcript was seen at 2.4 kb, as was a major transcript of approximately 1.4 kb. These data confirmed that the transfected cells expressed IL-2 while the parental NK-92 cells did not. The sizes of the IL-2 transcripts were confirmed three times in independent experiments. The size of the larger 2.4-kDa transcript seen in both NK-92MI and NK-92CI correlates with the expected length of hIL-2 transcript promoted by the 5' LTR and containing the *gag* and hIL-2 genes. No evidence of a longer RNA species was found in the NK-92CI cells, indicating that little or no gene expression was directed by the pCMV promoter of the pCEP4-LTR-hIL2 vector. The shorter transcripts seen in both NK-92MI and NK-92CI likely represent processed forms of the 2.4-kDa transcript, in which the packaging and part of the *gag* gene have been excised by a splicing event. The basis of the different hIL-

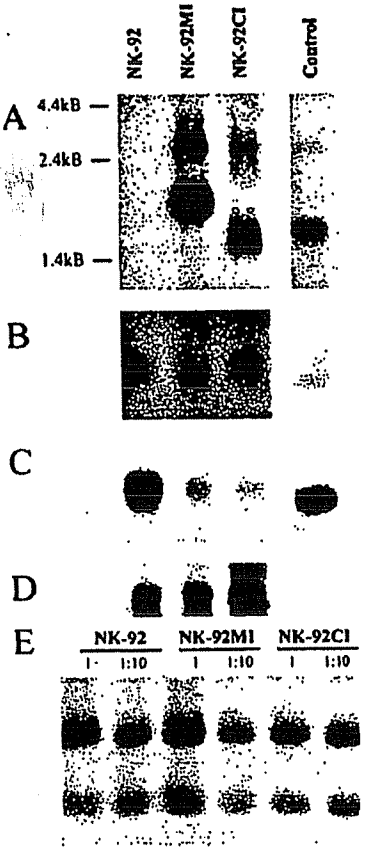


FIG. 3. Northern blot and RT-PCR analysis of cytokine and chemokine expression in NK-92, NK-92MI, and NK-92CI cells. RNA isolated from the parental and transfected cell lines was separated by agarose gel electrophoresis, blotted to nylon membrane by capillary transfer, and hybridized with probes for human IL-2 (A), TNF-α (B), lymphotactin (C), and β-actin (D). (A) No IL-2 expression was detected in NK-92. However, two RNA transcripts were detected in NK-92MI, with sizes of 2.4 and 1.9 kDa. Two transcripts were also detected in NK-92CI, with a major species of 1.4 kDa and a minor species at 2.4 kDa. A DNA fragment containing the human IL-2 cDNA was also run as a control. (B and C) Expression of TNF-α (B) and lymphotactin (C) was seen in NK-92, NK-92MI, and NK-92CI. cDNAs of TNF-α and lymphotactin were run as controls. (D) Expression of the housekeeping gene β-actin was used to control for amounts of mRNA. (E) To determine expression of the two closely related lymphotactin genes, SCM1α and SCM1β, RNA was subjected to RT-PCR and the products were digested with the restriction enzyme *Pma*CI, which cuts the SCM1β product while leaving SCM1α intact. PCR products were analyzed by Southern blot analysis with a probe for human lymphotactin. Expression of both SCM1α (456-bp PCR fragment) and SCM1β (226- and 230-bp PCR fragments) migrating as a single band) was detected in all NK-92 cell lines.

TABLE 1. SYNTHESIS OF HUMAN IL-2 BY NK-92 AND THE IL-2-INDEPENDENT VARIANTS NK-92MI AND NK-92CI[a]

Cell line	Day	Experiment 1	Experiment 2	Experiment 3	Average ± SD
NK-92	1	0	7	1	1.7 ± 3.8
	2	0	4	1	1.7 ± 2.1
	3	0	3	3	2.0 ± 1.7
NK-92MI	1	517	586	545	549.3 ± 34.7
	2	977	1462	1342	1260.3 ± 252.6
	3	1872	2610	2263	2248.3 ± 369.2
NK-92CI	1	7	13	8	9.3 ± 3.2
	2	14	16	17	15.7 ± 1.5
	3	22	18	23	21.0 ± 2.6

[a]One million NK-92, MI, or CI cells were cultured in 8 ml of IL-2-free Myelocult. Medium was harvested after 24, 48, and 72 hr and assayed for the presence of human IL-2 in the medium by ELISA. Levels are expressed as picograms of IL-2 per milliliter of medium.

2 transcript lengths in NK-92MI and NK-92CI is not clear. It is possible that the different vector constructs or the integration into the genome; in the case of NK-92MI, could have influenced transcript processing such as polyadenylation, capping, or splicing, thus leading to different mRNA lengths.

Levels of both primary and processed hIL-2 transcripts in NK-92MI indicated higher expression of the hIL-2 cDNA in NK-92MI cells than in the NK-92CI variant. One possible explanation for this is the presence of genetic elements in the pCMV promoter region of the pCEP4-LTR-hIL2 vector that negatively affect LTR promoter strength, resulting in reduced hIL-2 expression in NK-92CI. Certainly the converse is true, i.e., the presence of the LTR suppresses any pCMV directed transcription. Alternatively, the presence of enhancers or other promoting elements near the integration site of the MFG-hIL2 vector may have resulted in the enhanced gene expression seen in NK-92MI.

After confirming the expression of the IL-2 gene by Northern blot analysis, cells were assayed for production and secretion of hIL-2 by ELISA. Aliquots of 1 million NK-92, NK-92MI, and NK-92CI cells were plated in 8 ml of medium and supernatent was collected after 24, 48, and 72 hr for IL-2 analysis by ELISA. Background levels of IL-2 were detected in the supernatant of NK-92 cells at all three time points (2–3 pg/ml). Elevated IL-2 levels were detected in both NK-92MI and NK-92CI supernatents. (Table 1). NK-92MI produced much higher levels of IL-2 in comparison with NK-92CI, with levels ranging from 60 times higher after 24 hr (549.3 versus 9.3 pg/ml) to 80 times higher after 48 hr (1260.3 versus 15.7 pg/ml) and 72 hr (2248.3 versus 21.0 pg/ml).

To examine the effect of IL-2 transfection on other immunomodulatory molecules in these variants, TNF-α and lymphotactin expression was also examined using Northern blot analysis. Interferon γ expression was not analyzed as it is not expressed by NK-92 cells in response to IL-2 stimulation. Analysis of TNF-α in the NK cells revealed that all three lines expressed the cytokine gene. The TNF-α probe hybridized to a 1.6-kDa band in RNA isolated from NK-92, NK-92MI, and NK-92CI (Fig. 3B). TNF-α expression was slightly increased in both transfected cell lines relative to control, with slightly higher levels in NK-92MI after correction with B-actin (Fig. 3D). This was confirmed by ELISA, in which 1×10^6 cells were plated in 8 ml of medium and the supernatant was analyzed after 72 hr. NK-92MI showed elevated levels (19.4 pg/ml) versus NK-92CI (8.3 pg/ml) and parental cells (6.4 pg/ml with IL-2 at 150 U/ml and 0.7 pg/ml without IL-2). These results are consistent with induction of TNF-α expression following IL-2 stimulation of NK cells. RNA was also analyzed for expression of the chemokine lymphotactin, which has been reported to be expressed by a number of cell types including NK cells (Hedrick and Zlotnik, 1997) and this cell line (Henneman *et al.*, 1998). As with TNF-α, expression was detected in all three cell lines (Fig. 3C). A band of 1.35 kDa was seen in RNA from NK-92, NK-92MI, and NK-92CI. RT-PCR to analyze expression of the two closely related lymphotactin genes SCM1α and SCM1β indicated that both genes were expressed in the parental as well as the transfectanted cell lines (Fig. 3E). A 456-bp fragment and a 230-bp fragment, indicating SCM1α and SCM1β, respectively, were seen following RT-PCR analysis of RNA from NK-92, NK-92 MI, and NK-92CI. Analysis of gene expression indicates that lymphotactin expression was dramatically reduced in NK-92MI and was further decreased in NK-92CI. Therefore, introduction of the IL-2 gene to NK-92 cells resulted in expression of IL-2 in the transfectants. Expression of TNF-α and lymphotactin was maintained in transfected cells although the levels of TNF-α were slightly increased while those of lymphotactin were reduced.

Comparison of NK-92, NK-92MI, and NK-92CI

The parental NK-92 cell surface expression of a number of molecules has been examined (Gong *et al.*, 1994; G. Maki, unpublished data, 1998). These include CD1, CD2, CD3, CD4, CD5, CD8, CD10, CD11a (LFA-1), CD14, CD16, CD19, CD20, CD23, CD25 (IL-2 receptor, α chain), CD28, CD34, CD45, CD50 (ICAM-3), CD54 (ICAM-1), CD56, CD105 (ICAM-2), CD122 (IL-2 receptor, β chain), and HLA-DR. To compare the IL-2-independent transfectants with the parental cells, NK-92MI and NK-92CI were analyzed for CD2, CD3, CD4, CD8, CD10, CD11a, CD16, CD25, CD28, CD50, CD54, CD56, CD105, and CD122 expression by flow cytometric analysis. Analysis of the transfected cells revealed a pattern

identical to that seen on the untransfected parental cell line, with the exception of the IL-2 receptor α chain. The original report indicated that the receptor was expressed on the surface of NK-92 cells and that its expression was downregulated in response to IL-2 (Gong *et al.*, 1994). These results were initially confirmed by this work, in which NK-92 cells in unsupplemented medium had high levels of CD25 whereas use of medium supplemented with as little as 10 U of IL-2 per milliliter resulted in reduced expression. Analysis of the high IL-2-producing transfectant NK-92MI revealed decreased CD25 expression under all culture conditions examined (unsupplemented medium as well as in media with 10–1000 U of IL-2 per milliliter) while the low IL-2-expressing NK-92CI exhibited CD25 expression that was reduced in the presence of exogenous IL-2 at 10, 100, and 1000 U/ml. These results reporting downregulation of CD25 in response to IL-2 are identical to those reported by Nagashima and colleagues (1998) and are consistent with those originally reported for this cell line. However, subsequent investigations have been unable to distinguish between reduced IL-2 expression and inhibition of antibody binding to CD25 by IL-2. Extensive experiments with both the 2A3 (Becton Dickinson) and B1.49.9 (Beckman-Coulter) clones have indicated that the presence of IL-2 inhibits antibody binding and detection of the IL-2 receptor α chain, thus making it impossible to evaluate the IL-2 effects on CD25 levels, using these antibodies.

Functional assays

To compare proliferation of the parental and transfected cell lines, growth of NK-92, NK-92MI, and NK-92CI cells over 7 days was analyzed by cell counts using trypan blue exclusion. NK-92 cells plated into IL-2-supplemented medium, and NK-92MI and NK-92CI cells plated into IL-2-free medium, were all found to proliferate at similar rates. All cell lines achieved a similar maximum growth rate after an initial lag period with reduced growth. NK-92 experienced the shortest lag (approximately 24 hr), while NK-92MI and NK-92CI had similar lag periods of approximately 48 hr, although NK-92MI did consistently exhibit a slightly shorter lag time. However, by day 2, the growth rate was comparable for NK-92, NK-92MI, and NK-92CI. The extended lag for the transfected cells was probably due to the time required for NK-92MI and NK-92CI to condition the medium with sufficient amounts of IL-2 to support cell proliferation, while IL-2-supplemented medium was able to support NK-92 proliferation almost immediately. The higher IL-2 production from the NK-92MI variant probably also accounts for the slightly reduced lag period for these cells compared with NK-92CI. These observations were further confirmed when a similar pattern was seen on subculturing the cells in fresh medium after 3 days.

To evaluate the cytotoxicity of these transfected cells, a standard 4-hr ^{51}Cr release assay was performed to compare the toxicity of the parental cells, relative to NK-92MI and NK-92CI, against the standard test cell lines K562 and Raji. The levels of cytotoxicity of NK-92MI and NK-92CI were comparable to that seen with the parent cells (Fig. 4). Three to five independent experiments were conducted for each cell line to test the cytotoxicity of cells at effector-to-target ratios of 1:1, 5:1, 10:1, and 20:1. The cytotoxicity of both transfected cell lines against K562 and Raji was similar to that of the parental cells. The cy-

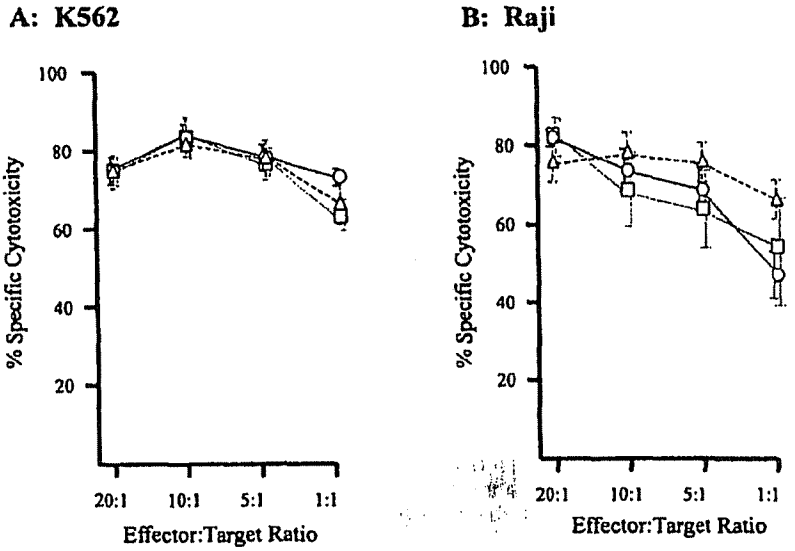


FIG. 4. Cytotoxicity of NK-92, NK-92MI, and NK-92CI against K562 and Raji target cells. The cytotoxic activities of the IL-2 transfectants were compared with that of the parental cell line. NK cells were mixed with ^{51}Cr-labeled K562 (A) or Raji (B) cells at 1:1, 5:1, 10:1, and 20:1 effector-to-target ratios for a 4-hr chromium release assay. The levels of cytotoxicity of NK-92 (○), NK-92MI (△), and NK-92CI (□) were compared. Transfection with the IL-2 gene did not affect cytotoxicity of the IL-2-independent variants. Cytotoxicity was comparable at all ratios for both K562 and Raji cells.

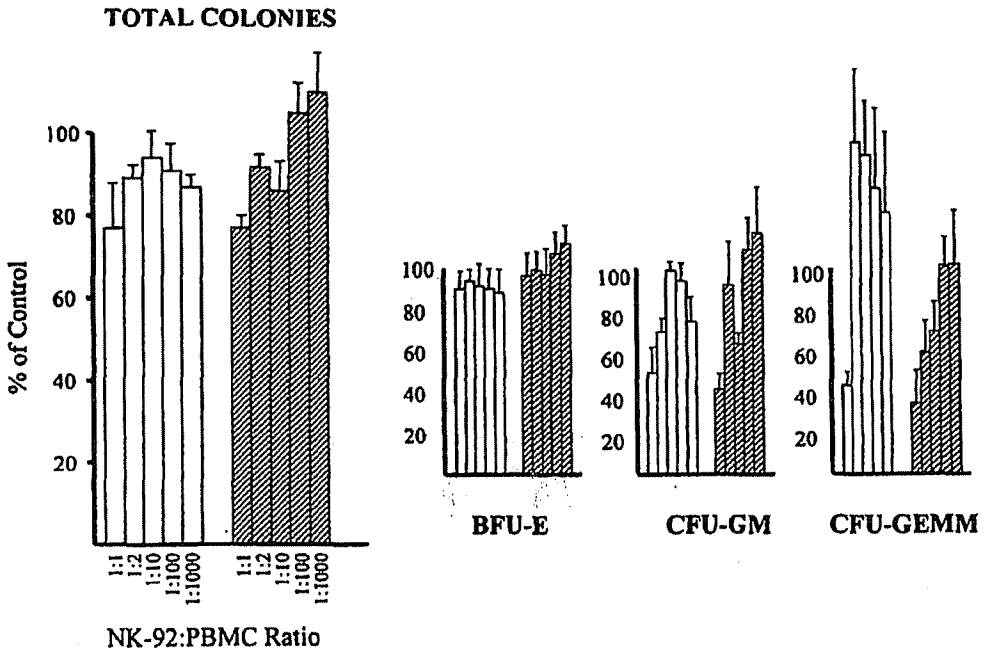


FIG. 5. Effect of NK-92MI and CI cells on hematopoietic progenitors. To assay the effect of the NK-92 cells on normal hematopoietic progenitors, a clonogenic assay was performed. Six hundred thousand normal PBMCs were incubated with 6×10^5, 3×10^5, 6×10^4, 6×10^3, and 6×10^2 irradiated NK-92MI or CI cells at ratios of 1:1, 1:2, 1:10, 1:100, and 1:1000 (NK:PBMC), respectively, for 48 hr. Cells were plated in methylcellulose at 2×10^5 cells per plate to give 10–100 colonies per dish after 14 days. Clonogenic output of PBMCs incubated with NK-92MI (open bars) and CI (hatched bars) is expressed as either total number of colonies or subclassified on the basis of colony type (BFU-E, CFU-GM, and CFU-GEMM). Overall, no significant effect of NK cells on clonogenic output was seen on total colony output and no consistent trends were seen. Similarly, negligible effects were seen in terms of effect of NK cells on BFU-E output. More pronounced effects were seen at higher ratios in CFU-GM and CFU-GEMM, although no consistent relationship was seen between clonogenic output and NK:PBMC ratios.

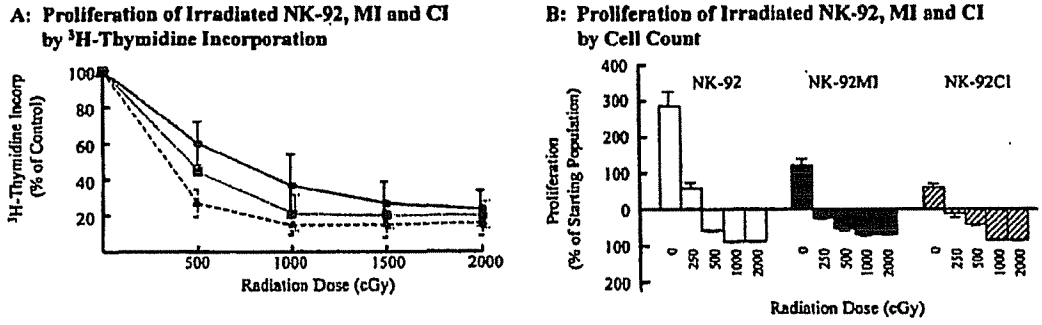
totoxicity of NK-92 against K562 ranged from 82 to 67%, while that of NK-92MI and NK-92CI ranged from 77 to 62% and from 82 to 62%, respectively. For Raji cells, NK-92 had 81 to 47% cytotoxicity, NK-92MI achieved 75 to 65% cytotoxicity, and NK-92CI showed a specific cytotoxicity of 82 to 52%.

One potential clinical application of the NK-92, NK-92MI, and NK-92CI cells is as an *ex vivo* purging agent for autologous stem cell grafts for stem cell transplantation. This would be aimed at eliminating any possible contaminating malignant cells that could be present in the graft following chemotherapy and stem cell harvest prior to reinfusion into the patient. For the NK cells to be suitable for such a purpose, they must be able to purge the malignant cells without killing the hematopoietic progenitor cells in the graft or influencing their hematopoietic potential. To assay this, a colony-forming cell (CFC) assay was performed in which the clonogenic output of hematopoietic progenitor cells in peripheral blood was examined following a 48-hr incubation with NK-92MI and NK-92CI at various ratios. Peripheral blood was selected because the majority of transplantations are now performed with peripheral blood stem cells. Parental NK-92 has previously been shown to have minimal effects on hematopoietic stem cells. In this work, both NK-92MI and NK-92CI also show little or no effect on clonogenic output. The number of total colonies following incubation with either NK-92MI or NK-92CI was similar to control, although a slight decrease was seen with the highest effector-to-PBMC ratio of 1:1 (Fig. 5). Total clonogenic output from both NK-92MI and NK-92CI was approximately 80% of control under this condition. However, no consistent trend was seen in terms of clonogenic output and the ratio of NK cells to PBMCs. In terms of specific colony types, there were no detectable differences in the number of output BFU-E colonies, which are the most numerous. Some effect was seen with both the CFU-GM and CFU-GEMM colonies. However, the absolute numbers of these colonies are low, making any conclusions difficult since small variations in the number of colonies have a large effect on the calculation of clonogenic output. An influence on CFU-GM and CFU-GEMM colonies is seen at higher ratios, but no consistent correlation between ratio and output was noted.

A major concern in any clinical application of these cells is the administration of a tumor cell line to patients. One possible solution is the use of radiation to control cell proliferation. The radiation dose required to control NK-92 proliferation ad-

equately has previously been established and the cells have been shown to maintain their cytotoxic activity following this treatment. To establish an appropriate dose for NK-92MI and NK-92CI, cells were irradiated at 250, 500, 1000, 1500 and 2000 cGy and assayed for proliferation by both [^{3}H]thymidine incorporation and cell counts (using trypan blue exclusion). From the [^{3}H]thymidine incorporation data, NK-92MI and NK-92CI proliferation was suppressed by a radiation dose of 500 to 1000 cGy whereas that of NK-92 was not (Fig. 6A). The level of thymidine incorporation reached a plateau at approximately 20% of levels seen with unirradiated control cells for NK-92CI and 10% for NK-92MI. From the cell count data, doses of 0 and 250 cGy of radiation were not sufficient to control proliferation of NK-92 cells (Fig. 6B). Exposure to 500 cGy, however, resulted in cell numbers remaining constant over 72 hr. Exposure to 1000 and 2000 cGy resulted in a 50–90% reduction in cell number over 72 hr. In contrast, exposure to 250 cGy of radiation was sufficient to control proliferation of both NK-92MI and NK-92CI according to the cell count data. Cell numbers remained consistent over 72 hr following exposure to this radiation dose. Irradiation with 500 cGy resulted in a slight decline in numbers while cells exposed to 1000 and 2000 cGy decreased substantially in number.

Under both assays, both NK-92MI and NK-92CI were found to be more sensitive to radiation than were the parental NK-92 cells. Proliferation of NK-92MI and NK-92CI was found to be more strongly suppressed than that of NK-92 at all radiation doses tested. Furthermore, a greater percentage of both



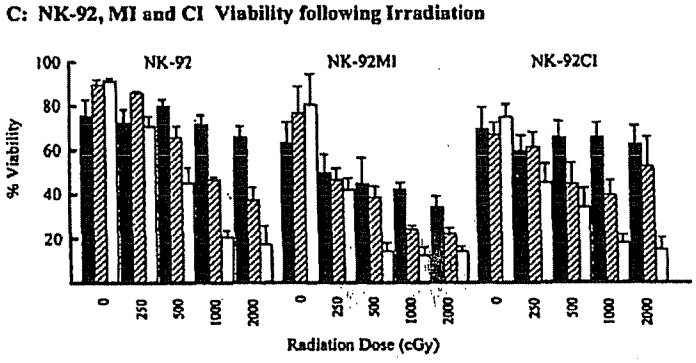


FIG. 6. Effect of irradiation on NK-92, NK-92MI, and NK-92CI proliferation and viability. To assess the effect of irradiation on the parental and transfected NK-92 cells, cells were exposed to 0-, 500-, 1000-, 1500-, and 2000-cGy doses of radiation and assayed for proliferation by a standard [^{3}H]thymidine incorporation assay. (A) Proliferation of NK-92 (●), NK-92MI (▲), and NK-92CI (■) is expressed as a percentage of [^{3}H]thymidine incorporation in control (unirradiated) cells. The radiation was least effective in inhibiting NK-92 proliferation at all doses. One thousand centigrays reduced proliferation to 40% of control, while 2000 cGY was only able to reduce it a further 10–15%. For NK-92MI and NK-92CI, proliferation reached a plateau of 15 and 20%, respectively, with a dose of 500–1000 cGy. No further reduction was seen with increased dose. NK-92CI appeared to be marginally more resistant than NK-92MI. (B) Proliferation of NK-92 (open bars), NK-92MI (solid bars), and NK-92CI (hatched bars) as determined by cell count 72 hr after irradiation, using trypan blue exclusion. Numbers of NK-92 cells were found to increase after exposure to 0 and 250 cGy. Doses of 500, 1000, and 2000 cGy resulted in a reduction in cell number after 72 hr. For NK-92MI and NK-92 CI, a dose of 250 cGy was sufficient to control proliferation. Five hundred, 1000, and 2000 cGy resulted in dramatic reductions in cell numbers. (C) To assay the effect of radiation on viability, NK cells were exposed to 0, 250, 500, 1000, and 2000 cGy of irradiation and assessed by trypan blue exclusion for viability after 24 hr (solid bars), 48 hr (hatched bars), and 72 hr (open bars). The relatively high radioresistance of NK-92 as compared with the IL-2 transfectants was also reflected in terms of viability. A higher percentage of NK-92 cells remained viable following irradiation at all doses and all times. Again, NK-92CI appeared to be slightly more resistant to irradiation than NK-92MI.

NK-92MI and NK-92CI were found to be directly killed by radiation, compared with the parental cells, at equivalent doses at judged by trypan blue exclusion. NK-92, NK-92MI, and NK-92CI cells were irradiated at 250, 500, 1000, and 2000 cGy and viability was determined 24, 48, and 72 hr following irradiation (Fig. 6C). The viability of NK-92 was higher than that of both transfectants at all dose rates tested. NK-92CI also showed consistently higher viability than did NK-92MI, although the difference between the two cell lines was relatively small. All of these data support a much higher sensitivity to radiation for the IL-2-independent variants. This is also reflected in the cytotoxicity of these cells following irradiation. Cells irradiated at 0, 1000, and 2000 cGy were tested after 3 days for cytotoxicity against K562 and Raji cells at effector-to-target ratios of 20:1, 10:1, 5:1, and 1:1. The cytotoxicity of NK-92 cells 3 days following irradiation at 1000 cGy was determined to be approximately 10–30% for K562 (Fig. 7A) and 30–50% for Raji (Fig. 7B). Irradiation at 2000 cGy resulted in a cytotoxicity level of 1–5% against K562 and 3–13% against Raji. In contrast, NK-92MI had only 0–5 and 0–1% cytotoxic activity against K562, and 0–1 and 0% against Raji, 3 days after exposure to 1000 and 2000 cGy of radiation, respectively. NK-92CI had only 1–4% cytotoxicity against K562 and 2–7% against Raji 3 days after irradiation at 1000 cGy; these levels fell to 0% against K562 and 0–2% against Raji after irradiation with 2000 cGy.

DISCUSSION

There has been substantial interest in the use of cytotoxic immune effector cells for adoptive immunotherapy of cancer. Attention has turned to the potential of autologous antigen-presenting cells to induce tumor-specific cytotoxic T lymphocytes. Other approaches, involving lymphokine-activated killer (LAK) cells and tumor-infiltrating lymphocytes (TILs), have also shown success in animal cancer models (Basse *et al.*, 1991; Crowley *et al.*, 1992) and clinical trials for advanced cancer (Rosenberg *et al.*, 1993, 1994). There has also been interest in NK cell therapy as a potential anticancer treatment. NK cells play roles in normal host immunosurveillance and anticancer mechanisms (Klein and Mantovani, 1993; Pross and Lotzova, 1993; Brittenden *et al.*, 1996). They are able to lyse malignant and virally infected cells without prior sensitization and their activity has been correlated with disease-free interval and overall survival of cancer patients (Hauch *et al.*, 1990; Brittenden *et al.*, 1996). Particularly relevant is the ability of NK cells to target cells with reduced or absent MHC class I expression (Klein and Mantovani, 1993; Pross and Lotzova, 1993; Brittenden *et al.*, 1996), which is the case with many malignancies (Garrido *et al.*, 1995), particularly in metastatic lesions (Cordon-Carlos *et al.*, 1991; Ferrone and Marincola, 1995; Restifo *et al.*, 1996). These data all suggest that NK cells may be an effective immunotherapy option, a hypothesis supported by strong *in vitro* (Porgador *et al.*, 1995, 1997; Cervantes *et al.*, 1996; Sillia *et al.*, 1996; Uharek *et al.*, 1996) and *in vivo* (Yasumura *et al.*, 1994; Okada *et al.*, 1996; Cesano *et al.*, 1997) data.

One obstacle to the clinical use of NK cells is the difficulty in expanding them *ex vivo*. Although progress has been made in large-scale expansion (Miller *et al.*, 1993; Silva *et al.*, 1995; Pierson *et al.*, 1996), it remains problematic and costly to obtain sufficient cell numbers for clinical use. An alternative is to use established cytotoxic NK tumor cell lines (Lu and Negrin, 1997), which would give access to large numbers of effector cells. This concept has been proved by Cesano *et al.* (1997), who showed that an NK-like cell line, TALL-104, was effective in treating a variety of malignancies in dogs.

NK-92 is a natural killer cell line that has been developed in

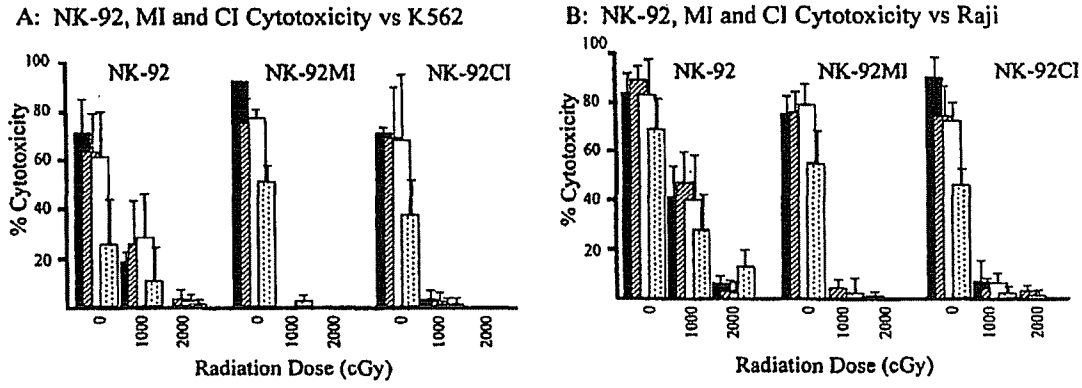


FIG. 7. Effect of irradiation on NK-92, NK-92MI, and NK-92CI cytotoxicity. To assess the effect of irradiation on the cytotoxicity of the NK cells, NK-92, NK-92MI, and NK-92CI were irradiated at 0, 1000 and 2000 cGy and tested after 3 days for cytotoxicity against K562 (A) and Raji cells (B). NK cells were tested at effector-to-target ratios of 20:1 (solid bars), 10:1 (hatched bars), 5:1 (open bars), and 1:1 (stippled bars). NK-92 cells maintained up to 30% cytotoxicity against K562 and 50% cytotoxicity against Raji when irradiated with 1000 cGy; this compared with 5% against K562 and 1% against Raji for NK-92MI, and up to 4% against K562 and 1% against Raji for NK-92CI. With a dose of 2000 cGy, NK-92 cytotoxicity was up to 5% for K562 and 13% for Raji. For NK-92MI, a cytotoxicity of 1% was seen against K562 and no activity was detected against Raji. NK-92CI was 2% effective against Raji and not toxic against K562 3 days after 2000 cGy of irradiation.

our laboratory from a patient with non-Hodgkins lymphoma (Gong *et al.*, 1994). It has a high cytolytic activity, even at low effector-to-target ratios, and is effective against a broad range of malignant target cells including cell lines derived from acute and chronic lymphoblastic and myelogenous leukemia (ALL, AML, CLL, CML), lymphoma, myeloma, melanoma, breast and prostate cancer, and neuroblastoma (Gong *et al.*, 1994; Yan *et al.*, 1998; Tam *et al.*, 1999). In studies by Yan *et al.* (1998), NK-92 cells were shown to have higher cytolytic activity and to kill a broader spectrum of malignant target cells than the TALL-104 cell line. Some work suggests that this high activity may be related, at least in part, to the lack of p58, an NK inhibitory receptor (KIR). NK-92 cells do not have detectable levels of p58 expression and reconstitution of KIR expression renders sensitive target cells resistant to NK-92 killing (Burshtyn *et al.*, 1996).

Studies to investigate the feasibility of using NK-92 as an immunotherapeutic agent have been encouraging. NK-92 is cytolytic to a large proportion of primary leukemic cells *in vitro* as well as *in vivo* (Yan *et al.*, 1998). NK treatment of SCID mice challenged with primary, patient-derived pre-B-ALL, T-ALL, and AML resulted in reduced tumor growth and increased survival. In another study using a SCID mouse–human melanoma model, NK-92 was also able to reduce tumor growth and increase survival of mice challenged with either primary or metastatic melanoma cell lines (Tam *et al.*, 1999). As well, NK-92 was found to be able to purge normal cells mixed with the erythroleukemic cell line K562 of the malignant cells (Klingemann *et al.*, 1996). NK-92 cells are able to specifically kill malignant cells with little or no influence on either normal hematopoietic progenitor cells (Klingemann *et al.*, 1996) or normal somatic cells (Tam *et al.*, 1999). Several other properties in addition to its high cytolytic activity and broad target range suggest that NK-92 may be a good candidate as an immunotherapeutic agent. These include easy *in vitro* expansion in static cultures or bioreactors, and growth in serum-free medium (Y.K. Tam, unpublished data, 1998). As well, since these cells maintain their cytolytic activity following irradiation, this can be used as a method to control NK cell proliferation during therapy (Klingemann *et al.*, 1996).

The cytotoxic activity of both primary NK cells and NK cell lines is dependent on cytokines such as IL-2 and/or IL-12 (Miller *et al.*, 1997). *In vivo* administration of IL-2 can induce NK/LAK activity (Miller *et al.*, 1997) but the level are limited by IL-2-associated toxicity (Weisdorf *et al.*, 1993), which results in suboptimal NK activation and cytotoxicity. Higher cytotoxic activity for immunotherapy can be achieved by *ex vivo* activation, but the effect of IL-2 withdrawal following infusion is unclear. IL-2 withdrawal could result in reduction of cytotoxicity and viability, leading to complete loss of activity (Miller *et al.*, 1993). Transfer of cytokine genes to provide an autocrine function has been described (Treisman *et al.*, 1995) and Miller *et al.* (1997) have reported the transfer of IL-2 genes into NK cells, which allowed for endogenous production of sufficient IL-2 to maintain proliferative and cytotoxic capacity.

NK-92 is dependent on IL-2 for viability, proliferation, and cytotoxicity. Earlier work has shown that the cells maintain cytotoxicity following IL-2 deprivation (Klingemann *et al.*, 1996) and most of the previously described data using these cells were gathered following IL-2 withdrawal. However, NK-92 cytotoxicity and viability diminish over time. Two potential shortcomings are that effective clinical cancer therapy may require prolonged treatment with the NK cells and that optimal cytotoxic activity most likely requires IL-2, in spite of the fact that the cells maintain some activity in its absence.

To address these concerns, we have described the introduction of two vectors containing the human IL-2 cDNA into NK-92 cells by particle-mediated gene transfer to create two stably transfected, IL-2-independent cell lines designated NK-92MI and NK-92CI. The presence of the IL-2 cDNA in these cell lines was demonstrated and both constitutively express and synthesize IL-2. Although the NK-92MI variant produced much higher IL-2 levels than did NK-92CI, endogenous production was sufficient to render both transfected cell lines independent of exogenous IL-2. Both variants were capable of long-term growth in IL-2-free medium. The availability of two IL-2-independent cell lines that produce different levels of IL-2 will allow optimization of the immunotherapeutic potential of these NK cells. If levels synthesized by NK-92MI cells are so high as to cause IL-2 toxicity, NK-92CI would be used. In addition, these cells may provide a dual benefit. In addition to their cytotoxic activity, treatment with NK-92MI or NK-92CI may also prove to be a suitable method by which to administer IL-2 systemically.

Comparison of the parental cells and the IL-2-independent variants indicated that transfection did not result in any alterations other than those properties associated with IL-2 expression. Analysis of NK-92, NK-92MI, and NK-92CI for a number of cell surface antigens indicated that expression was unaltered following transfection. Furthermore, expression of normally expressed cytokine and chemokine genes, specifically TNF-α and lymphotactin (both SCM1α and SCM1β), was maintained although levels of both varied in response to IL-2. As with the parental cells (Gong *et al.*, 1994), neither transfectant exerted a significant effect on clonogenic output from hematopoietic progenitors. Importantly, both NK-92MI and NK-92CI exhibited high cytotoxicity levels, comparable to that of the parental strain, when tested against standard target cells.

In the data reported here, IL-2 transfectants were more sensitive to radiation than was the parental strain, although the reasons for this are unclear. Proliferation of NK-92MI and NK-92CI variants was suppressed at lower radiation levels and radiation-induced lethality was much greater at equivalent doses relative to the parental cell line. Furthermore, the high IL-2-producing NK-92MI variant was more sensitive than the NK-92CI variant. One possible explanation for this may be that the production of IL-2 by NK-92MI and NK-92CI resulted in the activation of a number of genes, including those associated with cell proliferation, while the parental cells were irradiated under IL-2-free conditions. The higher sensitivity of activated and proliferating cells to radiation damage may account for the higher susceptibility of NK-92MI and NK-92CI to radiation damage and death.

As a result, a reduced level of irradiation would be sufficient to adequately control NK-92MI and NK-92CI proliferation while reducing lethality and inhibition of cytotoxicity. However, in spite of the ability to utilize lower doses, radiation may not be an ideal modality to control NK-92 proliferation for im-

munotherapy. Irradiated NK-92MI and NK-92CI cells exhibit reduced viability and cytotoxicity and have a limited effective life span, thereby reducing the advantages of IL-2-independent cells. Therefore, other approaches that would not affect NK viability, cytotoxicity, or life span but would allow selective control and elimination of these cells would be preferable. Alternatives include introduction of the herpes simplex virus thymidine kinase suicide gene for *in vivo* therapy or $CD34^+$ progenitor cell selection prior to reinfusion for *ex vivo* purging applications. Work is currently underway to assess the feasibility of such approaches.

To our knowledge, the generation of NK-92MI is the first report of stable genetic transfection using particle-mediated gene transfer, with the integration of the MFG-hIL2 vector into the NK-92 genome. In the case of NK-92CI, the absolute requirement of NK-92 cells for IL-2 provides ideal circumstances under which episomal vectors can be stably maintained without the need for selective agents. Other work describing stable transfection of NK-92 and a second cell line, YT-1, with hIL-2 by retroviral gene transfer has been reported (Nagashima *et al.*, 1998). These researchers describe results that are in agreement with those reported here, including the generation of IL-2-independent clones that maintain their high cytolytic activity. Furthermore, Nagashima *et al.* (1998) describe *in vivo* studies that demonstrate that these cells are able to prolong survival of mice with established liver metastases. We have described work supporting the effectiveness of these IL-2-independent NK-92 cells as immunotherapeutic agents. We evaluated the use of NK-92MI and NK-92CI for immunotherapy of human melanoma in a SCID mouse model (Tam *et al.*, 1999). A single treatment with irradiated NK-92MI and NK-92CI cells significantly increased both median and average life span of SCID mice challenged intravenously with primary and metastatic human melanoma cell lines. As well, in mice challenged subcutaneously with these same cell lines, NK-92MI and NK-92CI were able to delay tumor development and reduce tumor size compared with untreated mice.

In spite of the fact that NK-92MI and NK-92CI are genetically altered, transfection by particle-mediated technology offers advantages over retroviral gene transfer. Of particular concern in retroviral gene transfer to cells for human therapy is the potential for generation of replication-competent retroviruses by recombination of retroviral genes in viral producer cells (Martineau *et al.*, 1997; Wilson *et al.*, 1997). Furthermore, retroviral transduction involves integration of the viral genome into host genomic DNA, raising the possibility of insertional mutagenesis and malignant transformation of cells in a polyclonal, retrovirally transfected cell population (Verma and Somia, 1997). Particle-mediated transfer of the IL-2 gene to NK-92 cells alleviates many of these safety concerns.

In this study, we report the creation of two IL-2-independent NK-92 cell lines through the introduction of a cDNA encoding hIL-2: NK-92MI and NK-92CI. Although NK-92MI produces much higher levels of IL-2 compared with NK-92CI, both lines possess high cytotoxicity and similar growth characteristics. The transfected cells possess properties that make them excellent candidates for use in cellular immunotherapy. The introduction of the IL-2 gene provides the capacity for prolonged, optimal cytotoxic activity without the need for exogenous IL-2 support. We are currently evaluating the *in vivo* viability and cytotoxicity of these variants to determine if there is indeed an advantage in using the IL-2-transfected variants rather than the parental cells. Furthermore, these variants have been genetically transfected using particle-mediated gene transfer, a technology compatible with their use for human therapy. In this way, it may be possible to use these cells as an effective adoptive human cellular immunotherapy to achieve adequate killing of malignant cells, either *in vivo* to eliminate minimal residual disease, or *ex vivo* to achieve purging of an autologous graft.

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Phase I Clinical Trial with a Human Major Histocompatibility Complex Nonrestricted Cytotoxic T-Cell Line (TALL-104) in Dogs with Advanced Tumors[1]

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ABSTRACT

The human TALL-104 cell line is endowed with a uniquely potent MHC nonrestricted tumoricidal activity across several species. In view of the potential applicability of TALL-104 cells as an anticancer agent, this study was conducted to evaluate the possible toxicity and efficacy of this new cell therapy in a superior animal model with spontaneous tumors. Nineteen canine cases with advanced, refractory malignancies of various histological types were entered in the study. All dogs had failed all other available treatments and had very limited life expectancy. Cyclosporin A was administered p.o. (10 mg/kg/day) starting from the day before TALL-104 cell administration throughout the treatment to prevent rejection of the xenogeneic effectors. Lethally irradiated (40 Gy) TALL-104 cells (10^8/kg) were administered systemically following two treatment schedules. In the first schedule, the cells were given every other day for 2 weeks in a row and then once a week for 3 additional weeks; in the second schedule, TALL-104 cells were administered daily for a total of 5 days. None of the 19 cases showed significant clinical or laboratory toxicity; in addition, none of the dogs had to be withdrawn from the study because of immediate adverse reactions to the infusions. The severe side effects usually associated with classical lymphokine-activated killer therapy in association with high doses of interleukin 2, such as "capillary leak syndrome," were absent in this study. Remarkably, TALL-104 therapy induced various degrees of antitumor effects in 7 of the 19 dogs, including 1 complete response (continuing at +13 months), three partial responses (duration of 2 months, 3 months, and continuing at +2 months), and three transient responses. Clinical responses and immunological parameters correlated well in each case. Taken together, these data indicate that systemic administration of lethally irradiated TALL-104 cells in the absence of exogenous interleukin 2 may be regarded as a safe and promising adjuvant type of treatment for advanced cancer patients.

INTRODUCTION

This laboratory has developed a new cell therapy approach to cancer that might overcome some limitations associated with the transfer of a patient's own effector cells and might not require the concomitant administration of exogenous toxic cytokines, such as high doses of rhIL-2,[3] for efficacy. Our strategy is based on the use of a lethally irradiated clonal human T-cell line (TALL-104; $CD3^+$, $CD8^+$, $CD56^+$, and $CD16^-$) that is endowed with MHC nonrestricted killer activity against a broad range of tumors across several species, sparing cells from normal tissues (1–4). Both the cytotoxic function and the growth of TALL-104 cells are supported *in vitro* by rhIL-2. Adoptive transfer of γ-irradiated (40 Gy) TALL-104 cells into severe combined immunodeficiency mice has induced regression of transplantable human hematopoietic and nonhematopoietic tumors (5, 6). The same results were obtained in immunocompetent mice bearing syngeneic leukemia.[4] In the latter model, transfer of TALL-104 cells induced protective antitumor immunity in the host, as indicated by the ability of the cured mice to specifically reject additional tumor cell challenges. The overall data in both experimental mouse models with transplantable tumors prompted us to test the possible efficacy of this MHC nonrestricted killer cell line in a clinically relevant setting, such as the one provided by pet dogs with spontaneous tumors.

Cancer occurs in approximately 1 in 4 dogs annually. The types of tumors are similar to their human counterparts with regard to incidence, biological behavior, and response to therapy. Their spontaneous occurrence in this outbred population makes the dog an ideal model to study transitional forms of cancer therapy, especially those therapies that differ from conventional approaches and may not be immediately acceptable for use in humans.

As described in this report, repeated systemic administration of irradiated TALL-104 cells without exogenous IL-2 in terminally ill canine cancer cases resulted in remarkable clinical responses not associated with significant toxicity. Importantly, immunological parameters in these cases correlated well with clinical responses and laboratory data.

MATERIALS AND METHODS

Case Selection. Nineteen canine cancer cases were enrolled in a Phase I trial with γ-irradiated TALL-104 cells (Table 1). All cases had histologically documented cancers with evaluable or measurable disease and were under the care of K. A. Jeglum at the Veterinary Oncology Services (West Chester, PA). Eligibility criteria were: failed standard treatments; expected survival of >1 month; no central nervous system metastases; no antineoplastic therapy within at least 2 weeks prior to study entry; and no active systemic infection, coagulation disorders, or major cardiovascular or pulmonary disease. High *in vitro* susceptibility of the tumor biopsies to the lytic activity of TALL-104 cells, although not indispensable, was considered a plus in enrolling the dogs into the trial.

Pre-study evaluation included history and physical examination, complete blood cell count, serum chemistry profile, and appropriate imaging studies to measure disease (X-rays and ultrasounds).

Treatment Protocols. The initial 15 cases enrolled between November 1994 and June 1995 received γ-irradiated (40 Gy) TALL-104 cells at the constant dose of 10^8/kg in saline as i.v. bolus over 30 min, on alternate days for 2 weeks, followed by weekly injections for 3 additional weeks (a total of nine injections; Table 2, schedule I). The immunosuppressive drug CsA (Sandimmune, Sandoz, East Hanover, NJ) was administered at the dose of 10 mg/kg p.o. once a day, starting from the day before TALL-104 cell administration throughout the first 2 weeks of treatment. In the following 3 weeks, CsA was given to the dogs only the day before and on the same day of TALL-104 injection. Any objective antitumor response qualified the case for

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[3] The abbreviations used are: rhIL-2, recombinant human interleukin 2; CsA, cyclosporin A; LAK, lymphokine-activated killer; CR, complete response; PR, partial response; TR, transient response; PBMC, peripheral blood mononuclear cell; TNF, tumor necrosis factor; FACS, fluorescence-activated cell sorting; MKPS, modified Karnosky Performance Status; mAb, monoclonal antibody.

[4] A. Cesano *et al.*, submitted for publication.

Table 1 *Characteristics of the canine cases*

Number of patients	19
Median age (range)	7.5 (2–13)
Male/Female	11/9
Prior treatment	
None	0
Chemotherapy	7
Chemo + mAb therapy	9
Surgery	1
Chemotherapy and TNF-α	1
Disease histology	
Lymphoma	9
Melanoma	2
Mammary Adenocarcinoma	1
Squamous cell carcinoma	2
Hemangiosarcoma	1
Malignant histiocytosis	3
Mast cell tumor	1
Disease sites	
Lung	5
Spleen	6
Liver	4
Mediastinal	4
Lymph nodes	10
Skin	3
Bone marrow	1
Heart	1
Chest	1
Abdomen	2
CNS	2
Maxilla	2
Nose	2
Bone	2
Ears	1
Muscle	2

another treatment cycle. Based on *in vitro* data (see below), some canine cases received OKT3 mAb at the dose of 0.1 $\mu g/10^6$ cells.

Because of the lack of significant immunosuppression observed in cases treated according to schedule I (all 15 dogs developed anti-human antibodies within 10–14 days after the first TALL-104 injection, see below), 5 additional cases enrolled between June and December 1995 were treated with a modified protocol that consisted of daily administration of CsA (5 mg/kg, twice a day) and TALL-104 cells (10^8/kg i.v.) for 5 consecutive days (Table 2, schedule II).

Protocol Modifications and Toxicity Monitoring. When needed, the protocols described above were modified by withholding TALL-104 cell injections, rather than by dose reduction. This happened very seldom and was mostly due to newly emerging medical reasons unrelated to the cell therapy but was most frequently associated with disease progression or with the immunosuppressive regimen. In a dog with melanoma (F. M.) and one with nasopharyngeal carcinoma (L. B.), who received TALL-104 cells intralesionally following schedule I, some injections were withheld due to massive tumor necrosis and bleeding resulting from such injections (see below).

Clinical signs of acute toxicity (such as fever, chills, hypotension, diarrhea, vomiting, and others) were monitored during and after cell infusion. The majority of the dogs were treated as outpatients, and the owners were properly instructed to report on the well being of their pets during cell therapy. Complete blood cell counts and serum chemistry profiles were performed on blood and serum samples obtained from the cases before study and before each cell administration.

Response Criteria. Standard response criteria were used. A CR was defined as the complete disappearance of the tumor for at least 4 weeks with no new lesions developing. PR was defined as 50% or greater reduction in the sum of the products of the largest perpendicular diameters of all measurable disease and maintained for at least 4 weeks without any new lesions appearing. Mixed response was defined as the simultaneous regression and progression of disease at different sites of involvement. Progressive disease was an increase of greater than 25% in the sum of the products of the largest perpendicular diameters or the appearance of new lesions. Stable disease was defined as disease not meeting the above criteria for response or progression. Response duration was measured from the date when the response was first documented and was updated through the date of submission of this manuscript. Response duration was required to be ≥4 weeks to qualify for CR, PR, or mixed response. Tumor shrinkage of >25% lasting less than 4 weeks was referred to as a TR of biological rather than clinical interest.

Large-Scale Expansion of TALL-104 Cells for Therapy. TALL-104 cells were maintained in humidified incubators at 37°C with 10% CO_2 in endotoxin-free Iscove modified Dulbecco's medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Sigma Chemical Co., St. Louis, MO) and 100 units/ml rhIL-2 (a gift from Dr. Maurice Gately, Hoffman-La Roche, Nutley, NJ). T175 vented-cap flasks (Falcon, Franklin Lakes, NJ) were used for the large expansion of the cytotoxic cells. The cultures were microscopically inspected every day, and the cell density was adjusted to 1×10^6 cells/ml. *Mycoplasma* contamination was checked weekly using a commercial PCR kit (American Type Culture Collection, Rockville, MD).

At the time of therapy, the cells were harvested, centrifuged in 250 ml conical centrifuge tubes (Corning, New York, NY) at 1500 rpm for 10 min, washed twice in saline (Abbott Laboratory, King of Prussia, PA), resuspended in 100 ml saline, and transferred into a blood transfer pack (Baxter Diagnostics, Inc., Glendale, CA) for the i.v. infusion. Aliquots were removed from the bag for evaluation of cell-mediated cytotoxicity and sterility (quality control). The cells were injected into the dogs within 2–4 h from irradiation. OKT3 mAb was directly added to the infusion bag just prior to injection when we had *in vitro* evidence that the dogs' tumors were resistant to lysis by TALL-104 cells alone but were killed efficiently by such cells upon triggering of their CD3/T-cell receptor (see below).

Cytotoxicity Assays. The cytotoxic activity of TALL-104 cells against freshly explanted canine tumor biopsies and the established canine lymphoma cell line Pilgrim (standard positive control) was measured in an 18-h ^{51}Cr release assay, as described (1, 2, 4). A fixed number (10^4/well) of ^{51}Cr-labeled target cells was tested against four effector cell concentrations. PBMCs were isolated from the cases blood by Accu-Prep lymphocytes (Accurate Chemical, Westbury, NY) gradient centrifugation and tested for cytotoxicity in an 18-h ^{51}Cr release assay against their own tumor cells (when available), TALL-104 cells, and the Pilgrim cell line.

Cytokine Assays. TALL-104 cells (1×10^6/ml) were incubated at 37°C for 24 h, either alone or with fresh tumor samples (E:T ratio, 5:1), in the presence and absence of OKT3 mAb (0.1 μg/ml). Cell-free conditioned media were harvested, filtered, and tested for the presence of human IFN-γ, TNF-α, and TNF-β, using cytokine-specific ELISA kits (Endogen, Boston, MA) according to the procedure specified by the manufacturing company. The sensitivity of the assay was 20 pg/ml for IFN-γ and TNF-α and 8 pg/ml for

Table 2 *Treatment schedule*

Schedule of administration	Days of week	Immunosuppression regimen (daily)	Dose of cells	Total no. of injections (range)
I 15 cases		CsA (10 mg/kg)	10^8/kg ±OKT3 (0.1 $\mu g/10^6$cells)	9 (4–17)
Week 1	M-W-F			
Week 2	M-W-F			
Weeks 3–6	W			
Rest; clinical evaluation				
II 4 cases		CsA (5mg/kg BID[a])	10^8/kg ±OKT3 (0.1 $\mu g/10^6$cells)	5 (5–10)
Week 1	M→F (daily)			
Rest; clinical evaluation				

[a] BID, twice a day.

Table 3 *Correlation between laboratory and clinical findings*

Case	Diagnosis	*In vitro* sensitivity to TALL-104 cell lysis[a]	No. of TALL-104 infusions	Plasma levels of human[b]			Generation of antibodies to TALL-104 cells[c]	Generation of CTL against TALL-104 cells[c]	Clinical response[d]
				TNF-α	IFN-γ	TNF-β			
H. M. C.	Lymphoma (st-V)	NT	4	−	−	±	N	N	PD
D. M.	Lymphoma (st-III)	NT	6	±	−	±	Y	N	PD
C. L.	Lymphoma (st-IVB)	NT	9	−	−	++	Y	N	PD
M. H.	Lymphoma (st-IVB)	−	6 without OKT3	−	−	±	Y	Y	PD
		+++	6 with OKT3	−	+	+	Y		MR
Y. A.	Lymphoma (st-IV)	+++	4	−	±	±	Y	N	PD
D. L.	Lymphoma (st-III)	NT	5	−	−	+	Y	N	TR
L. S.	Lymphoma (st-IVA)	NT	5 with OKT3	±	±	NT	N	N	PD
F. M.	Melanoma	++++	4 intratumoral	−	−	+	Y	N	TR
P. B. K.	Malignant histiocytosis	NT	8	−	−	+	Y	N	CR
L. H.	Mammary adenocarcinoma	++++	8 + 9	−	+	+	Y	Y	PR/SD
C. C.	Squamous cell carcinoma	NT	12 with OKT3	±	+	+	Y	N	PR/SD
L. B.	Squamous cell carcinoma	++	8 intratumoral and i.v.	−	−	±	Y	NT	PD
N. R.	Lymphoma (st-IVB)	NT	5 with OKT3	−	−	−	Y	N	PD
S. L.	Hemangiosarcoma	NT	4	−	−	−	N	NT	PD
B. B.	Malignant histiocytosis	−	7	−	±	+	Y	N	PD
B. C.	Mast cell tumor	+++	10	−	−	+	N	Y	TR
R. C.	Lymphoma extra-nodal	+	10 with OKT3	−	−	−	Y	NT	PD
F. C.	Malignant histiocytosis	−	11	−	+	++	Y	Y	PR
M. F.	Melanoma	++++	5	−	−	±	Y	Y	PD

[a] % TALL-104 cell lysis at E:T ratio 10:1. ++++, ≥80%; +++, ≥50%; ++, >30% but <50%; +, >10% but <30%; −, <10%; NT, not tested.
[b] −, <20 pg/ml for TNF-α and IFN-γ, and <8 pg/ml for TNF-β; ±, >20 pg/ml but <50 pg/ml; +, >40 pg/ml but <100 pg/ml; ++, >100 pg/ml.
[c] Y, yes; N, no.
[d] PD, progressive disease; SD, stable disease; MR, mixed response; NE, not evaluable; PR, partial response; TR, transient response.

TNF-β. The plasmatic levels of TALL-104-released IFN-γ, TNF-α, and TNF-β were evaluated during therapy in serial serum samples of the canine cases.

Immunological Monitoring. Serum and PBMC samples taken before study and before each TALL-104 cell injection were monitored for the development of humoral and cellular immune responses, respectively, against TALL-104 cells. Sera were diluted at 10^{-3} in FACS buffer (Ca^{2+}- and Mg^{2+}-free PBS, with 0.1% NaN_3, and 2% IgG-free horse serum) and transferred into a 96-well, round-bottomed plate (Falcon). TALL-104 cells were washed in FACS buffer and added to the plates at 10^5/50 μl/well. Sera and cells were incubated for 1 h at room temperature and washed three times with FACS buffer. FACS buffer was used as a negative control. A FITC-conjugated rabbit anti-dog IgG (whole molecule; Sigma) was added at 2×10^{-2} for 1 h at 4°C. At the end of the incubation, the cells were washed, resuspended in 150 μl FACS buffer, and analyzed by flow cytometry using an Ortho cytofluorograph cell sorter. The development of TALL-104-specific cellular immune responses was monitored by testing the cytotoxic activity of the dogs PBMCs against ^{51}Cr-labeled TALL-104 cells in an 18-h ^{51}Cr release assay (1, 2, 4).

PCR Analysis. The presence and persistence of irradiated TALL-104 cells in the dogs' blood were evaluated by PCR analysis of frozen DNA aliquots extracted from their PBMCs at various intervals. Two primers specific for the human minisatellite region YNZ.22 (7) were used. An oligonucleotide probe recognizing 24 nucleotides in the middle of the amplified sequence was used to demonstrate the specificity of the PCR products by Southern blot hybridization (7).

OKT3 Preparation. OKT3 hybridoma cells (American Type Culture Collection) were expanded at 37°C in 5% CO_2 in Iscove's modified Dulbecco's medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal bovine serum. Cells were injected (10^7 i.p.) into 6-week-old BALB/c mice (Taconic, Germantown, NY) pretreated 7 days earlier with Pristane (Sigma; 0.5 ml i.p.) and sublethally irradiated (400 rads) just prior to the hybridoma cell injection. After 10–12 days, ascites fluids were collected, pooled, and purified using an IgG affinity chromatography column (mAb-Trap GII; Pharmacia, Uppsala, Sweden). The purified mAb was checked for reactivity against TALL-104 cells by immunofluorescence analysis, filtered on 0.2-μm pore-size filters (Corning), and stored in aliquots at −20°C.

RESULTS

Case Profile. All 19 dogs enrolled in this study had histologically confirmed malignancies, including lymphoma (9 dogs), metastatic breast adenocarcinoma (1 dog), malignant histiocytosis (3 dogs), naso-pharyngeal squamous cell carcinoma (2 dogs), metastatic hemangiosarcoma of the liver (1 dog), oral melanoma (2 dogs), and mast cell tumor of the leg (1 dog; Table 1). All cases had refractory disease and had failed standard treatments, such as surgery and chemotherapy. The nine dogs with lymphoma had received chemotherapy and immunotherapy with CL/mAb 231 (a murine anti-canine lymphoma antibody; Refs. 8 and 9). rhTNF-α had been given to the dog with a mast cell tumor in addition to prednisone. The dog with metastatic breast cancer had been subjected to surgical removal of the primary mammary tumor mass; no cytotoxic treatment for pulmonary metastasis had been given to this dog, based on the knowledge that metastatic breast cancer in canines is refractory to chemotherapy (10). All treatments were discontinued at least 1 week before enrollment in the TALL-104 study.

Treatment Protocol. The number of infusions given to each dog varied from 4 to 17 in schedule I and 5–10 in schedule II (Tables 2 and 3). The total number of cells infused in each dog ranged from 4×10^9 to 10^{11}. Four dogs with lymphoma (M. H., L. S., N. R., and R. C.) and one with naso-pharyngeal squamous cell carcinoma (C. C.) received OKT3 mAb together with TALL-104 cells (Table 3). In two cases (R. C. and M. H.), this decision was based on *in vitro* studies demonstrating a higher killer activity of the cells against the dogs' tumor sample upon activation of their CD3/T-cell receptor (Fig. 1 and data not shown) and/or higher levels of lymphokine production by TALL-104 cells incubated overnight with the tumor cells plus OKT3 (data not shown). In the other three cases (L. S., N. R., C. C.) in which tumor biopsies could not be obtained for *in vitro* testing (Table 3), OKT3 was given in the attempt to overcome an expected tumor resistance to TALL-104 cell killing, suggested by previous *in vitro* observations with ^{51}Cr-labeled canine fresh tumors of the same histological type (Fig. 1).

Clinical Laboratory Changes and Toxicity Associated with TALL-104 Therapy. A complete list of treatment-related toxicities is shown in Table 4. No case had to be withdrawn from the protocols due to reactions to the infusions. No life-threatening acute reactions were observed during and after TALL-104 cell therapy, except for an isolated anaphylactic-like reaction seen in a dog with lung metastatic breast cancer (L. H.) during the second cell injection (schedule I); the animal collapsed 5–10 min after the start of the infusion, showing weak pulse and decrease in capillary refill time. The infusion was

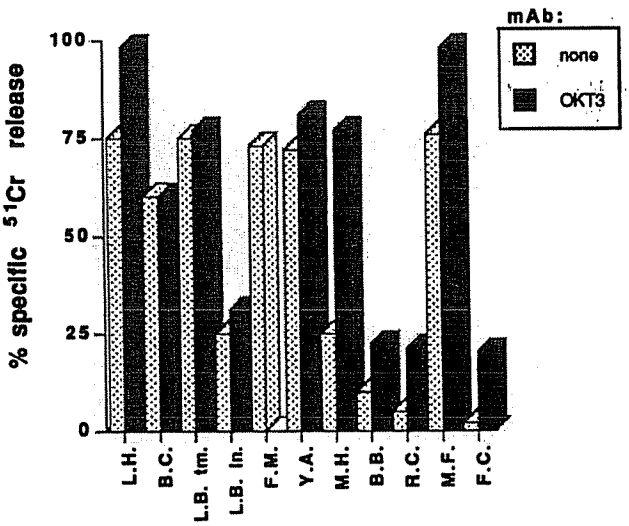

Fig. 1. Cytotoxic activity of irradiated TALL-104 cells against fresh tumor biopsies from canine cases as tested in an 18-h ^{51}Cr release assay. The E:T ratio was 50:1. The assay was done in the presence and absence of OKT3 mAb (0.1 μg/ml). *L. H.*, dog with breast carcinoma; *B. C.*, dog with mast cell tumor; *L. B.*, dog with squamous cell carcinoma; *F. M.* and *M. F.*, two dogs with melanoma; *Y. A.*, *M. H.*, and *R. C.*, three dogs with lymphoma; *B. B.* and *F. C.*, two dogs with malignant histiocytosis. *tm.*, tumor mass; *ln.*, lymph node.

immediately stopped, and the dog was treated with dexamethasone i.v. Once the dog was stabilized, the cell infusion was completed at a slower rate. After this episode, the dog was premedicated with dexamethasone just before every TALL-104 cell injection and did not show any further adverse reaction during the rest of the treatment. Interestingly, this dog was also the case that, in this study, received the highest number of cells and a total of 17 injections (Table 3).

Mild vomiting, responsive to antiemetics, and diarrhea were seen in five cases. Given their time of appearance, these symptoms seemed to be related to CsA administration rather than to cell infusion. Some abnormalities in liver (increased transaminases, alkaline phosphatases, and hyperbilirubinemia), and renal (increased blood urea nitrogen and creatinine levels) functions were seen sporadically (Table 4). Discrete leukocytosis with neutrophilia was noted in 11 cases; the number of WBCs increased rapidly after the infusion (reaching a level of 2 to 5 times the baseline level, with 98 to 99% neutrophils) and returned to normal within 24–48 h after halting the therapy.

All together, these data indicate that large numbers of irradiated TALL-104 cells (up to 10^{11} over 5 weeks) can be safely infused into canine cases with advanced cancers.

Clinical Responses. Various degrees of antitumor effects were observed in seven of the 19 cases enrolled in this study (Tables 3 and 5). In particular, one dog achieved a CR, three dogs showed a PR/stable disease, and three had TR in specific lesions, indicated by significant tumor necrosis (Table 5). The other 12 cases continued to progress with their disease during the treatment.

The Scottish terrier who achieved a CR (P. B. K.) presented with end-stage malignant histiocytosis. His prognosis was grave due to the inability to treat with doxorubicin (because of cardiac toxicity) and lack of response to other chemotherapeutic agents. When enrolled into the TALL-104 trial (schedule I), the dog had numerous s.c. metastatic lymph nodes, and the MKPS was 2. A PR was achieved after six cell infusions, and a complete regression of all lesions was observed after the eighth injection. At that time, the performance status was drastically improved (MKPS, 0). Two weeks after the end of TALL-104 therapy, new s.c. nodules appeared on the left front and hind legs, the malignant nature of which was confirmed by needle aspirates. Surprisingly, the nodules spontaneously regressed within 48 h from their appearance. Immunological studies performed on P. B. K.'s blood showed the presence of a highly activated immunological status just at the time when regression of the tumor nodules took place (Fig. 2*A*), as indicated by the acquired ability of his PBMCs to spontaneously lyse a human tumor target (K562). Cytotoxicity returned to baseline (neg-

Table 4 *Laboratory changes and clinical toxicity in 19 cases*

Fever/chills	0
Vomiting	5
Diarrhea	5
Anaphylactic-like reaction	1 (only 1 infusion of 17)
Diffuse erythema/pruritus	0
Weight gain	0
Anorexia	1
Dyspnea	0
Lethargy	2
Infections	0
Hypotension	0
CNS disturbance	0
Cardiac arrhythmias	0
Death (pulmonary insufficiency)	0
Abnormal liver functions:	
Increased ALT[a]	6
Increased Al Ph	1
Hyperbilirubinemia	1
Abnormal renal functions:	
Increased blood urea nitrogen	1
Increased creatinine levels	1
Anemia	0
Thrombocytopenia	0
Leukopenia	0
Eosinophilia	0
Leukocytosis with neutrophilia	11

[a] ALT, alanine aminotransferase; Al Ph, alkaline phosphatase.

Table 5 *Characteristics of the responding cases*

Treatment with irradiated TALL-104 cells	Case	Age/Sex	Tumor (site)	*In vitro* sensitivity to TALL-104 cell killing	No. of TALL-104 injections (schedule)	Clinical response[a]	Duration
Without OKT3	D. L.	13/M	Lymphoma (st. III) (spleen, liver, LN)	NT[b]	5 (I)	TR	NE
	F. M.	11/M	Melanoma (oral/nasal)	++++[c]	4 (I)	TR	NE
	P. B. K.	8/M	Malignant histiocytosis (skin, lymph nodes)	NT	8 (I)	CR	+13 months
	L. H.	5/F	Mammary adenocarcinoma (lung)	++++	8 + 9 (I)	PR/SD	2 months
	B. C.	12/F	Mast cell (leg)	++++	10 (II)	TR	NE
	F. C.	7/M	Malignant histiocytosis (skin, lymph nodes)	−	11 (II)	PR	+2 months
With OKT3 (0.1 μg/10^6 cells)	C. C.	11/F	Squamous cell carcinoma (nose)	NT	9 (I)	PR/SD	2 months

[a] TR, transient response; CR, complete response; PR, partial response; SD, stable disease.
[b] NE, not evaluable.
[c] Percentage of lysis in a scale from <1 (−) to ≥80% (++++) at the E:T ratio of 10:1.

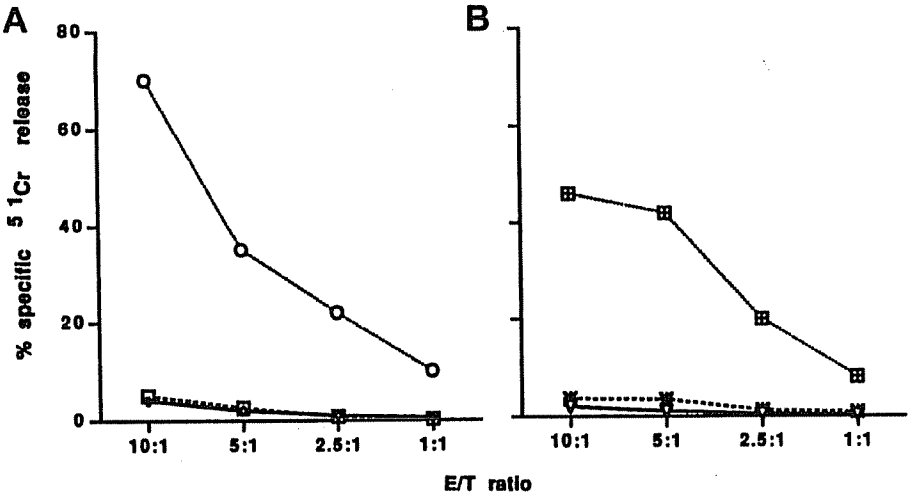

Fig. 2. *A*, cytotoxic activity of PBMC from P. B. K. (a dog with malignant histiocytosis who achieved durable CR) against K562 cells. PBMCs were harvested and tested in an 18-h ^{51}Cr release assay before cell therapy (□), during spontaneous remission of the relapsed s.c. lesions (○), and 2 weeks later (◇). *B*, cytotoxic activity of PBMCs taken 2 weeks after completion of TALL-104 cell therapy from L. H. (a dog with breast carcinoma who achieved PR) against K562 cells (•), TALL-104 cells (▽), and cells from the primary tumor (⊞) as measured in an 18-h ^{51}Cr release assay. The lytic activity of L. H.'s PBMCs against all three targets before cell therapy was <1%.

ative) levels 2 weeks after regression of the relapsed nodules. P. B. K. remains a complete responder at the time of this writing (13 months after CR was achieved).

One of the three partial responders (L. H.) had multiple metastatic lung lesions from primary anaplastic mammary adenocarcinoma, poor prognosis due to the early age of disease onset (5 years old), and rapid development of metastases. After 2 weeks of cell treatment (schedule I), the case showed a 50% reduction of the original tumor mass, which remained stable for 2 months. During that time, an active immune response (not present at the beginning of the treatment) against her own tumor could be demonstrated by *in vitro* cytotoxic assays using her PBMCs as effectors (Fig. 2*B*). Ten weeks after completion of cell therapy, new metastatic lung lesions appeared, and another treatment cycle with TALL-104 cells did not result in clinical improvement. Interestingly, immunological tests at this time demonstrated the presence of a specific immune response (both cellular and humoral) against TALL-104 cells (Table 3). This dog is still alive (7 months after cell therapy) with progressive metastatic disease. The second case who experienced a PR was a poodle (C. C.) with invasive squamous cell carcinoma of the nasal passage with extensive intranasal disease and bone lysis (MKPS = 1) at the start of cell therapy. The dog was treated with TALL-104 cells plus OKT3 following schedule I. Also in this case, a PR was achieved after 2 weeks of cell treatment with significant improvement in the dog's well being. The disease remained stable during the following 8 weeks but finally progressed with appearance of central nervous system metastasis. The third case who had a PR was a West Highland white terrier (F. C.), who presented with multiple recurrent skin lesions. The diagnosis of malignant histiocytosis analogous to the P. B. K. case was confirmed by cytology and specific immunohistochemical analysis of needle aspirates. In the 4 weeks following TALL-104 cell therapy (schedule II), the nodular lesions on the metatarsus progressively regressed, but the intradigital lesions and the popliteal lymph node stayed unchanged. The dog was boosted twice (within a 4-week period), each time with three injections of TALL-104 cells (10^8/kg/day). After the second boosting, the interdigital lesions also regressed, and the lymph node shrank to <50% of its initial volume. This is where the disease stood 2 months later.

Of the three cases that displayed TR (Table 5), one was a Doberman pinscher (F. M.) who presented with a very extensive melanoma mass along the left maxillary gengiva extending onto the hard palate and nasal sinus. Intratumoral injections of TALL-104 cells (into multiple sites of the tumor) resulted in a rapid (within 24 h from the first injection) and remarkable necrotic response, with tissue loss and severe bleeding (Fig. 3). The protocol was modified by withholding some injections. Because of the dog's poor clinical progress, the owner elected euthanasia. The second case was a Scottish terrier (D. L.) with lymphoma, presenting with multicentric lymphoadenopathy and hepatosplenomegaly. The dog had been treated aggressively with chemotherapy without any response, and his life expectancy was not more than one month (MKPS, 1). After two injections of TALL-104 cells (schedule I), needle aspirates showed severe necrosis of the peripheral lymph nodes. Cytological examination of a needle aspirate revealed neutrophils and RBCs with no lymphoma cells visible (Fig. 4). Repeated lymph node aspirates showed severe abscessation of all peripheral nodes and the absence of infectious agents. After five cell injections, the owner, discouraged by the poor clinical progress of the dog, elected euthanasia. The third case was a mixed-breed dog (B. C.)

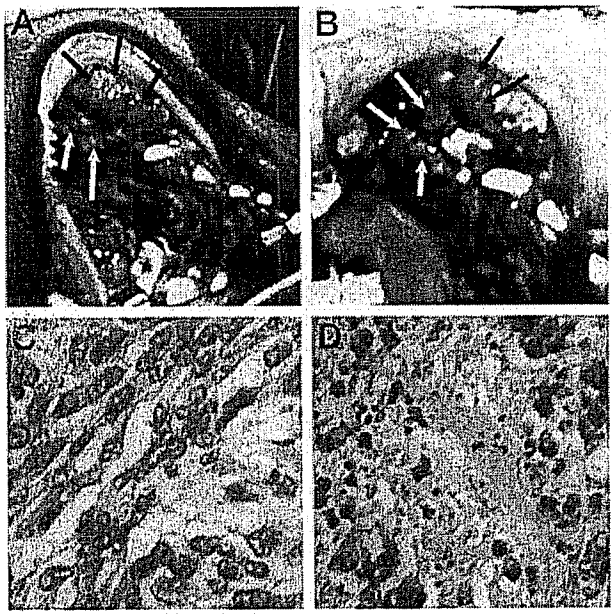

Fig. 3. *A* and *B*, oral cavity of F. M. (a dog with oral melanoma who experienced TR) during intralesional TALL-104 cell therapy. *Black arrows*, live tumor-growing tissue; *white arrows*, necrotic tissue. Note the increasing extension of necrotic tissue when the therapy was halted (*B*). *C* and *D*, microscopic appearance at the site of injection of the tumor before (*C*) and after (*D*) TALL-104 therapy. H&E staining, ×400.

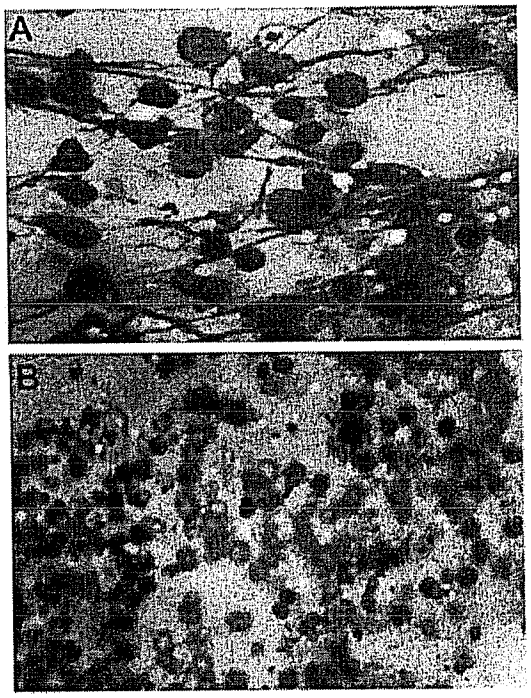

Fig. 4. *A*, needle aspirate from the submandibular lymph node of D. L. (a dog with lymphoma who experienced TR) before TALL-104 cell therapy. Note the presence of round, "healthy" lymphoma cells in the preparation. *B*, needle aspirate from the same lymph node at the end of the first week of systemic TALL-104 cell therapy (three injections); RBCs, granulocytes, and macrophages are the only detectable cells in the preparation, together with cellular debris. Giemsa staining, ×400 (*A*) and ×200 (*B*).

with mast cell tumor masses on the right hind leg (one mass on the lateral aspect of the femur and one on the medial aspect of the stifle), non-resectable and highly infiltrating (Fig. 5*A*). The month before TALL-104 cell therapy (schedule II), the dog received prednisone, benadryl, and rhTNF-α; cytokine administration was immediately discontinued because of its severe side effects. Interestingly, during each TALL-104 cell injection and in the following hour, clinical evidence of mast cell degranulation (edema erythematous of the leg) was observed (Fig. 5*B*). About 40% reduction of the tumor masses with resolution of the lymphoedema was achieved during cell therapy (Fig. 5, *C-E*). Unfortunately, the dog suddenly died 2 days after halting the therapy for what appeared to be a thromboembolic accident, the nature of which (tumor or clot) was not determined.

Correlation between Laboratory and Clinical Findings. Studies performed to evaluate possible correlations between laboratory and clinical findings, indicative of TALL-104 cell therapeutic efficacy and/or toxicity, are reported in Table 3. Although the number of cases treated was too small to draw statistically significant correlations, the following conclusions could be drawn: (*a*) the number of TALL-104 cell infusions in this Phase I study did not seem to be predictive of clinical efficacy. Although some of the responder dogs received higher numbers of injections (>9), clinical and biological responses were usually seen during the first cycle of therapy and, in some cases, after the first infusion (D. L., F. M., and B. C.). Moreover, responder dogs were also the ones given higher cell doses because they qualified for boosts; (*b*) more predictable of treatment efficacy seemed to be the presence of human TNF-α, IFN-γ, and TNF-β in the sera of the treated dogs (Table 3). Six of the seven responder cases had significant levels (ranging from 40 to 150 pg/ml) of at least one of the cytokines tested (usually TNF-β) in sequential testings, whereas detectable plasma levels of these cytokines were observed only occasionally in 4 of the 12 nonresponders; (*c*) *in vitro* sensitivity of the tumors to TALL-104 cell killing was not always a good indicator of clinical responses. Although the tumor biopsies of three of the four responder cases for which tumor specimens were available were highly susceptible to TALL-104 lysis *in vitro* (alone or after OKT3 stimulation; Fig. 1 and data not shown) and/or induced high levels of cytokine production in TALL-104 cells (data not shown), it is also true that five of six tumor specimens tested from nonresponder cases displayed sensitivity to TALL-104 cell killing *in vitro* (Table 3).

Immune Response against TALL-104 Cells. Despite the immunosuppressive regimen with CsA, 16 cases developed a humoral immune response against TALL-104 cells, usually between days 10 and 14 from the beginning of the treatment (see Table 3 and nine examples in Fig. 6). However, none of the TALL-104 reactive sera inhibited TALL-104 killer activity against the standard canine lymphoma target Pilgrim in *in vitro* cytotoxicity assays (data not shown). Cellular immune responses against TALL-104 cells could be demonstrated in 5 of the 19 treated dogs (Table 3; two examples are given in Fig. 7).

Detection of Circulating TALL-104 Cells. PCR amplification of the human minisatellite region YNZ.22 was performed on the cases' PBMCs at different intervals before, during, and after cell therapy to document the time of appearance and the kinetics of disappearance of TALL-104 cells from the circulation. Based on *in vitro* data (11), the lethally irradiated cells were expected to survive in the cases' blood for a maximum of 48–72 h. An example of PCR analysis with one dog (M. H.) is given in Fig. 8. Using this technique, circulating TALL-104 cells could almost always be detected during the treatment, even in the dogs that were treated intralesionally (data not shown). However, 1 week after completion of TALL-104 therapy (*i.e.*, after the last transfer) circulating TALL-104 cells were no longer detectable by PCR in any of the dogs examined.

DISCUSSION

The TALL-104 cell line is endowed with a uniquely potent tumoricidal activity across MHC barriers, yet sparing cells from normal tissues (1–6). The major objective of the present study was to evaluate

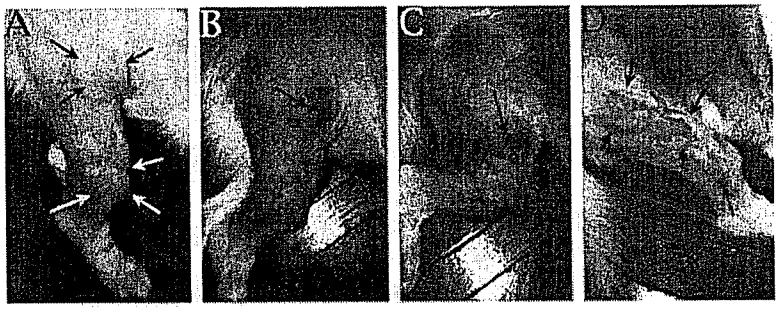

Fig. 5. *A*, macroscopic appearance of the s.c. tumor masses on the right hind leg of B. C. (a dog with mast cell tumor who experienced TR) before TALL-104 cell therapy. *Black arrows*, femoral mass; *white arrows*, stifle mass. *B* and *C*, appearance of the same lesions during TALL-104 cell treatment; note the erythematous color of the skin. *Arrow*, appearance of a necrotic center in the femorous mass. *D*, appearance of the s.c. tumors at the end of a 2-week cell therapy; note the total resolution of lymphoedema and the reduction (>40%) of both lesions (*arrows*).

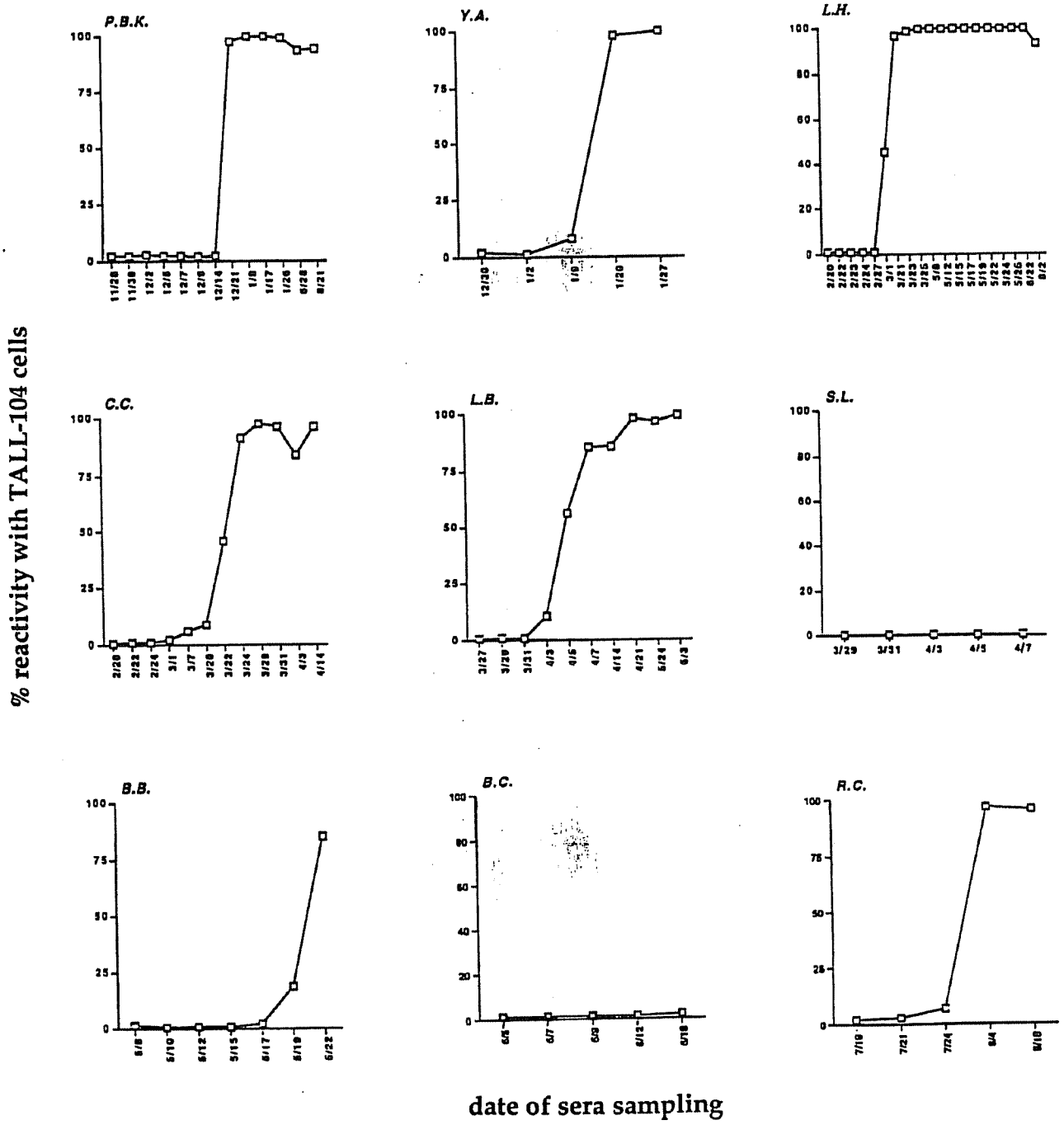

Fig. 6. Humoral immune responses mounted by nine treated dogs against TALL-104 cells. Sera of the treated dogs were harvested at the indicated dates in 1994 and 1995 and diluted 10^{-3} to test for the presence of TALL-104-specific antibodies.

the potential toxicity associated with systemic administration of lethally irradiated, nondividing, TALL-104 cells in immunocompetent animals, such as dogs, with naturally occurring malignancies. In addition, it was of interest to correlate any potential toxicity and clinical response with immunological laboratory findings.

Before embarking in this Phase I clinical trial, our data in three healthy dogs (School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA) injected with a single dose of TALL-104 cells (10^8/kg i.v.) had shown the absence of clinical and laboratory toxicity, except for a transient (8–24 h) and slight increase in liver transaminases that was not associated with alterations in alkaline phosphatase or bilirubin levels.[5] In the present study, abnormalities in liver function were seen in about 30% of the treated dogs; however, their correlation with TALL-104 cell therapy was sometimes difficult to assess because of progressive disease involving the liver parenchyma. In the few cases in which these alterations could be ascribed to TALL-104 cell injections, they were always limited to transami-

[5] A. Cesano, S. Visonneau, J. Wolfe, A. Gillis, R. J. O'Reilly, and D. Santoli, manuscript in preparation.

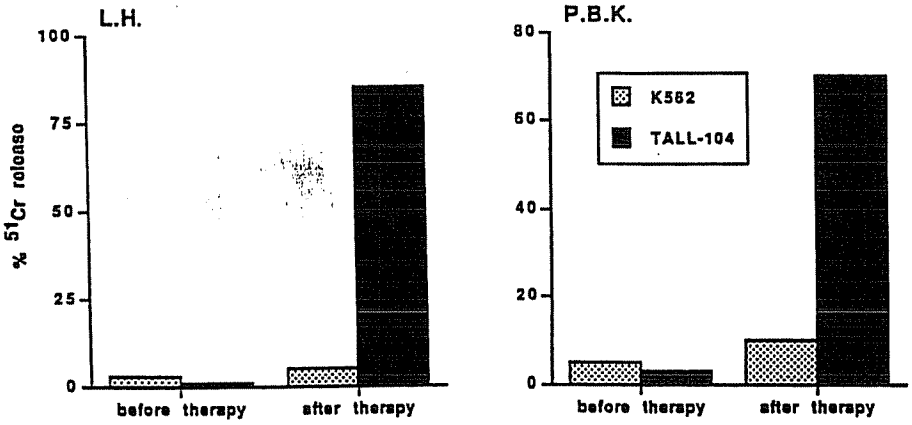

Fig. 7. CTL responses against TALL-104 cells generated by two TALL-104 cell-treated dogs. PBMC samples were harvested before and 1 month after completion of cell therapy and were tested for killer activity against K562 and TALL-104 cells in an 18-h ^{51}Cr release assay. The E:T ratio was 50:1.

nases, were moderate (increases ranged from 2 to 5 times the normal levels), transient, and completely reversible upon termination of therapy. Another type of toxicity (mild, grade 1–2) observed during TALL-104 cell treatment in <5% of the treated dogs was gastrointestinal (vomiting and diarrhea). This has also been reported to occur in 80% of the cases during LAK/IL-2 therapy in humans (12). Gastrointestinal symptoms in the dogs were easily controlled with drugs and, given the time of their appearance, seemed to be more related to CsA administration than to cell infusion.

No severe toxicities (grade 3 or 4), such as hypotension and oliguria secondary to the increased capillary permeability, were observed in our study. These effects have been reported to be induced by conventional LAK/IL-2 therapy (13). Although the mechanism by which IL-2 and LAK cells induce a vascular leak syndrome is unknown, LAK cells have been shown to bind to and lyse normal human vascular endothelial cells *in vitro* (14, 15). Whether this applies also to TALL-104 cells has not been tested. However, the absence of vascular leak syndrome in mice, dogs, and more recently, monkeys treated with TALL-104 cells in the absence of exogenous IL-2,[5] suggests that this effect does not reach biological significance with TALL-104 cells. Similar to our findings, LAK/IL-2 therapy has been reported to frequently alter hepatic functions (serum transaminases, alkaline phosphatase, and total bilirubin; Ref. 16). It has been suggested that, in general, IL-2-activated lymphocytes are hepatotoxic; the strong correlation between peak lymphocyte counts achieved after IL-2 priming and serum transaminase levels supports this contention (17). As in the case of TALL-104 cells, the LAK cell-induced liver abnormalities returned to baseline values after completion of the therapy, indicating that the insult to the liver was transient and reversible (16). None of the hematological abnormalities induced by LAK/IL-2 therapy (anemia, lymphopenia followed by rebound lymphocytosis, eosinophilia, and thrombocytopenia with coagulation disorders; Ref. 18) were detected in the present study. Interestingly, leukocytosis with neutrophilia was seen in about 50% of the treated dogs; it was of fast onset (detectable within 24 h from cell injection), moderate (2–3 times the normal counts), and rapidly reversible once therapy was halted (24–48 h). The same phenomena have been observed in mice and monkeys injected with high TALL-104 cell doses.[5] Although the cause of the observed neutrophilia postinjection is not clear, it is very likely related, at least in part, to the high levels of granulocyte-macrophage colony-stimulating factor production by TALL-104 cells in response to tumors (3). In this respect, human granulocyte-macrophage colony-stimulating factor has been shown to be cross-reactive with several species, including canines (19).

The only "life-threatening" toxicity observed in our studies was an isolated episode of anaphylactic-like reaction easily controlled by steroids. This was the type of toxicity we were most concerned about before initiating this trial because of the use of xenogeneic effectors. Moreover, throughout the course of this study, we could exclude the induction of "chronic" or "late" side effects in the treated dogs, including TALL-104-induced leukemia, based on the lack of persistence of these cells in the circulation, demonstrated by PCR.

CsA failed to prevent the development of humoral immunity against TALL-104 cells. The daily dose of CsA (10 mg/kg) could not be increased because this drug was not well tolerated by the dogs. Moreover, its use together with other immunosuppressant drugs, such as steroids, is not acceptable because steroids have negative effects on the tumoricidal and cytokine release functions of TALL-104 cells (data not shown). We are now investigating the possibility of doing cell therapy in the absence of immunosuppressants by taking advantage of the 10-day "window" needed by the dogs' immune system to mount a humoral response against TALL-104 cells from the start of the therapy. We envision that if the treatment has to be repeated at a later time, when an immune response against TALL-104 cells has been established, a short-term immunosuppression regimen with azathioprine could be implemented just before the second round of cell therapy. This idea is based on our recent *in vitro* data showing no effects of azathioprine on TALL-104 cell functions and its ability to significantly diminish established humoral and cellular responses against TALL-104 cells *in vivo*.[6] Ideally, TALL-104 cell boosts for 1–2 days could be performed without immunosuppressants.

No reports to date have shown clinical and laboratory correlations in association with LAK/IL-2 therapy in humans; in particular, neither the *in vitro* activity of LAK cells nor the LAK cell dose infused was found to be predictive of clinical efficacy or toxicity (16). The mean total number of LAK cells infused per patient ranged in different clinical trials from 5.6×10^9 to 5.1×10^{10} cells (20). In our study, the maximum number of TALL-104 cells injected was double (10^{11}). A

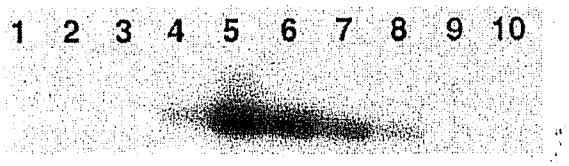

Fig. 8. PCR amplification of the human minisatellite region YNZ.22 performed on PBMCs from a dog (M. H.) before and at different times after TALL-104 cell injection. *Lane 1*, molecular weight; *Lane 2*, water; *Lanes 3–9*, 0, 2, 4, 8, 24, 48, and 72 h after TALL-104 cell injection; *Lane 10*, 5 days after TALL-104 cell injection.

[6] A. Cesano, S. Visonneau, and D. Santoli, unpublished observations.

correlation between clinical response to IL-2 therapy and sustained production of TNF was reported in a study by Blay *et al.* (21), in which TNF concentrations in the serum of the patients during IL-2 infusion ranged from 5 to 130 pg/ml. Our study in pet dogs confirm this correlation between clinical response and serum levels of human TNF. In addition, Moore *et al.* (22) were able to induce clinical responses in some dogs with spontaneous tumors by using escalating doses of rhTNF (up to the maximally tolerated i.v. dose of 125 mg/m^2) in conjunction with rhIL-2. The clinical responses were transient and associated with minimal toxicity, primarily gastrointestinal, similar to our study.

An important feature in our study is the demonstration that TALL-104 cell treatment elicited in the dogs the generation of both MHC nonrestricted cytotoxic cells, capable of killing the classical natural killer-sensitive human tumor target K562, and of tumor-specific CTL. These cellular activities correlated very well with the clinical responses observed. PBMC from four of the five responder dogs tested developed MHC nonrestricted killer activity against human tumor targets (Fig. 2), whereas only 2 of the 10 nonresponders tested did so. These observations support the possibility that the tumor regression noted in these responders was mediated not only by a direct lytic effect of TALL-104 cells but also, indirectly, through the recruitment of the host immunity and production of cytokines. This contention is also supported by the observation that durable clinical responses were achieved slowly and continued for a long time after the cell therapy was halted. Moreover, the spontaneous regression of relapsed nodules documented in the case of P. B. K. strongly suggests that TALL-104 therapy might have triggered the generation of protective antitumor immunity, similar to a vaccine approach. This intriguing possibility supports our recent observations in a leukemic mouse model in which upon TALL-104 therapy the cured animals rejected a second high-dose leukemic cell challenge.[4]

Surprisingly, the sensitivity of the dogs' tumors to TALL-104 cell lysis *in vitro* did not appear to be a good indicator of clinical responses. The interpretation of this observation is a complex issue since multiple additional factors play an important role in antitumor efficacy, in particular the tumor burden at the time of initiation of therapy and the accessibility of the tumor to the infused cells. It is noteworthy that some of the owners reported an increased level of activity, well being, and alertness in their dogs during TALL-104 therapy, even when no dramatic responses could be documented clinically.

Based on the advanced clinical stage and heavy pretreatment of the case population used in this study, the antitumor responses observed were unexpected. It is reasonable to assume that in dogs with a smaller tumor burden, the efficacy of TALL-104 cells would be greater, as indicated by our own murine studies where tumor load at the time of treatment was crucial for therapeutic success (5, 6).[7] In this respect, the finding that the dogs with lymphoma who represented ~50% of the treated population did not display significant clinical responses to TALL-104 therapy suggests that lymphomas might not be a good candidate for immunotherapy with TALL-104 cells; alternatively, the heavy chemoimmunotherapeutic treatment received by the dogs with lymphoma in our study might have rendered their tumor resistant to the xenogeneic effectors.

TALL-104 cell treatment is now being pursued as a single agent in Phase II/III trials in canine cancer cases with minimal residual disease, after remission induction, who have a high risk of relapse. Because of the lack of TALL-104 cell toxicity, the possibility of combining this cell therapy with other chemotherapeutic agents is also under investigation in this laboratory using both *in vitro* and *in vivo* approaches.

[7] A. Cesano, S. Visonneau, and D. Santoli, unpublished data.

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ORIGINAL ARTICLE

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Toxicological and immunological evaluation of the MHC-non-restricted cytotoxic T cell line TALL-104

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Abstract The human MHC-non-restricted cytotoxic T cell line TALL-104 has been shown to display potent antitumor effects in several animal models with spontaneous and induced malignancies. In view of its potential future use in cancer therapy, we investigated the tolerability and target-organ toxicity of these cells in various animal species. The acute toxicity of TALL-104 cell administrations was evaluated in: (a) healthy immunocompetent mice and immunodeficient (SCID) mice bearing human tumors using multiple (up to 15) intraperitoneal (i.p.) injections, and (b) healthy dogs, tumor-bearing dogs, and healthy monkeys using multiple (up to 17) intravenous (i.v.) injections. TALL-104 cells were γ-irradiated (40 Gy) prior to administration to mice and dogs, but administered without irradiation in monkeys. Cell doses ranged from 5×10^7/kg to 10^{10}/kg for each injection. All regimens were well tolerated, the main clinical signs observed being transient gastrointestinal effects. Moderate and transient increases in liver transaminase levels were observed in all animal species. Discrete and transient leukocytosis with neutrophilia was also noted in dogs and monkeys after i.v injections of TALL-104 cells. Histological analysis revealed foci of hepatic necrosis with lympho-/mono-/granulocytic infiltration in immunocompetent mice injected i.p. with 5×10^9–10^{10} cells/kg. In the same mice, the colon showed an increased number of muciparous cells and alterations in the villi structure: these alterations were completely reversed by 72 h after the last injection, while liver alterations reversed more slowly (1 week). No delayed or chronic toxicity was observed in any of the animals even when non-irradiated TALL-104 cells were administered: both immunocompetent mice and healthy dogs were found to be grossly and histopathologically normal when sacrificed (1 year and 1 month after the last TALL-104 injection respectively). TALL-104 cells did not persist in these hosts. In addition, monkeys showed no molecular signs of TALL-104-cell-induced leukemia in their blood 1 year after the last cell injection. Despite immunosuppression, most of the tumor-bearing dogs as well as the healthy dogs and monkeys developed both humoral and cellular immune responses against TALL-104 cells. The data derived from these preclinical studies suggest that administration of high doses of irradiated TALL-104 cells is well tolerated and would be unlikely to induce severe toxicity if applied in clinical trials to the treatment of patients with refractory cancer.

Key words Cell therapy · MHC-non-restricted cytotoxic T cell line · Acute toxicity · Chronic toxicity · Biodistribution · Immunological effects · Hematological effects

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Introduction

Clinical trials have demonstrated the antitumor efficacy of lymphokine-activated killer (LAK) cells in conjunction with recombinant human interleukin-2 (rhIL-2) in patients with renal cell cancer and melanoma [1–3]. However, this therapy has also produced significant toxic side-effects, largely ascribed to the administration of IL-2, which can lead to capillary leak syndrome and, in turn, life-threatening anasarca and multi-organ system dysfunction [1–6]. Although completely reversible upon termination of IL-2 therapy, the increased vascular permeability has led to varying degrees of interstitial pulmonary edema during treatment, producing major respiratory compromise in

some cases [7]. Unfortunately, the antitumor effects of IL-2 and its toxicity are both dose-related; this constitutes an important drawback to its clinical use. Numerous phase I and II clinical trials have been conducted to evaluate different routes of administration [8] and dose schedules [9, 10] for IL-2, and combinations of IL-2 with other cytokines [11–14], drugs [15–18], or cells (LAK, tumor-infiltrating lymphocytes) [19–21] in efforts to minimize the side-effects of IL-2 administration without compromising its potency as an antitumor agent. Thus far, these studies have yielded promising but inconclusive results.

We have developed a new cell-therapy approach to cancer that might overcome the limitations of LAK/IL-2 therapy because it does not require the concomitant administration of exogenous cytokines such as IL-2 for efficacy. This approach is based on the use of the IL-2-dependent human leukemic T cell line TALL-104 (CD3/TCR$\alpha\beta^+$, $CD8^+$, $CD16^-$), which was established and characterized in our laboratory [22–25]. These cells are endowed with MHC-non-restricted killer activity against a broad range of tumors across several species, while sparing cells from normal tissues [22–25]. Tumor cell lysis by TALL-104 cells occurs by a perforin-mediated pathway or can be Fas-dependent. Moreover, cytokines released by TALL-104 cells upon contact with tumor targets [such as interferon γ (IFNγ), tumor necrosis factor (TNF)α, TNFβ and transforming growth factor (TGF)β] exert cytostatic effects on tumor cell growth. We have previously shown that γ irradiation (40 Gy) does not significantly affect TALL-104 cell cytotoxic activity and cytokine secretion [26]. Irradiated TALL-104 cells are very effective in purging bone marrows from leukemic cells both in vitro and in immunocompetent mouse models [27, 28]. When used in adoptive-transfer experiments, γ-irradiated (40 Gy) TALL-10^4 cells induced regression of transplantable hematopoietic and nonhematopoietic tumors in mouse models and of spontaneous cancers in dogs [29–31].

In the present study, we investigated the tolerability and potential target organ toxicities of irradiated and non-irradiated TALL-104 cells administered i.p. and i.v. into animals of different species.

Materials and methods

Cell lines

Human tumor cell lines (erythroleukemia K562, glioblastoma U87-MG, lung carcinoma A549, melanoma WM451, prostatic carcinoma DU-145) were purchased from American Type Culture Collection (Rockville, Md.) and maintained in Iscove's modified Dulbecco's medium (IMDM; Gibco BRL, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, Ga.; complete medium). The TALL-104 cell line was also maintained in complete medium supplemented with 100 U/ml rhIL-2 (Chiron, Emeryville, Calif.) in a humidified incubator with 10% CO_2. All cell lines were repeatedly monitored for *Mycoplasma* contamination.

Table 1 Schedule of γ-irradiated TALL-104 cell administration in tumor-bearing SCID mice

Dose of γ-irradiated TALL-104 cells (kg^{-1})	Number of mice	Schedule of i.p. administration
10^9	20	Daily for 15 days
5×10^9	80	Daily for 10 days
10^9	80	Alternate days (total of 6×); weekly (total of 6×)

OKT3 preparation

OKT3 hybridoma cells (American Type Culture Collection) were expanded at 37 °C in 5% CO_2 in complete medium. Cells were injected (10^7 i.p.) into 6-week-old BALB/c mice (Taconic, Germantown, N.Y.) pretreated 7 days earlier with Pristane (Sigma; 0.5 ml i.p.) and sublethally irradiated (40 Gy) just prior to the hybridoma cell injection. After 10–12 days, ascites fluids were collected, pooled and purified using an IgG affinity chromatography column (mAb-Trap GII; Pharmacia, Uppsala, Sweden). The purified mAb was checked for reactivity against TALL-104 cells by immunofluorescence analysis, filtered on 0.2-μm-pore-size filters (Corning, New York, N.Y.) and stored in aliquots at −20 °C.

Mice

Six-week-old Balb/c mice (Charles River Laboratories, Wilmington, Mass.) were housed in The Wistar Institute Animal Facility in micro-insulator cages. Mice were injected i.p. with γ-irradiated TALL-104 cells (40 Gy) at a dose of 5×10^9/kg twice a day (at 8-h intervals) or at the single dose of 10^{10}/kg (10 mice per group). Mice were checked daily for clinical signs of toxicity. Blood was drawn by retro-orbital puncture with heparinized capillary tubes (Fisher Scientific, Pittsburgh, Pa.) 24 h and 1 week after the last cell injection. Complete serum chemistry screenings (including alanine aminotransferase, aspartate aminotransferase, albumin, blood urea nitrogen, creatinine, glucose, total bilirubin, Na, K, Cl) were performed. Some sera were also tested for the presence of human cytokines (see below). Two mice per group were sacrificed at 24 h and 72 h and organs were removed for histopathological analysis. The remaining mice were maintained for analysis of long-term toxicity.

Five- to 6-week-old CB-17/SCID mice (Charles River Laboratories), housed in a pathogen-free environment in The Wistar Institute Animal Facility, were engrafted s.c. with the human tumor cell lines listed above. At different times after tumor cell transfer (from day 1 to day 14), mice were injected i.p. with γ-irradiated TALL-104 cells

Table 2 Laboratory tests performed on dog and monkey blood samples before, during, and after TALL-104 cell injection

Whole blood	Sera
Red blood cells	Glucose
White blood cells	Blood urea nitrogen
Differential	Creatinine
Hematocrit	Phosphorus
Hb	Calcium
Mean corpuscolar volume	Potassium
Mean corpuscolar Hb concentration	Chloride
Mean corpuscolar Hb	Carbon dioxide
Platelets	Total protein
	Albumin
	Alanine aminotransferase
	Alkaline phosphatase
	Total bilirubin
	Cholesterol
	Anion gap

Table 3 Toxicity studies in non-human primates; schedules of administration of TALL-104 cells. *M, W, F* Monday, Wednesday, Friday

Schedule			Monkey no.	Immunosuppression daily		Dose of TALL-104 cells (kg^{-1})	
				Cyclosporin A (5 mg/kg twice daily)	Methylprednisolone (0.5 mg/kg twice daily)	Non-irradiated	Irradiated
I	week 1	M	1	+		10^8	
	week 2	M				10^8	
	week 17						10^7 (boost)
II	week 1	W, F, M	2	+	–	$2.5-5\times10^8$	
			3	+	–	$2.8-5\times10^8$ + OKT3 ($0.1\ \mu g/10^6$ cells)	
	week 10						10^7 (boost)
III	week 1	M→F (daily)	4	+	+	$5\times10^7-5\times10^8$	
	week 4						10^7 (boost)
	week 1		5	+	+		
	week 4						10^7 (boost)

following the doses and schedule of administration detailed in Table 1. Blood was collected at various intervals (24 h after a single injection and 1–2 weeks after the last injection of a cycle). Sera were separated by centrifugation and the presence of human cytokines was tested in the pooled sera (see below). Some mice were sacrificed at different times after the last TALL-104 cell injection (see Results) and subjected to necropsy. Some mice that appeared cured from their tumors upon TALL-104 cell therapy were maintained for 1 year and thereafter sacrificed for histopathological evaluation of long-term toxicity (see below).

Biodistribution of TALL-104 cells in Balb/c mice

TALL-104 cells (2.5×10^7/mouse) were labeled overnight in a 37 °C humidified 10% CO_2 incubator with 0.25 mCi $Na_2[^{51}Cr]O_4$ (DuPont NEN, Boston, Mass.). After three washes in IMDM, cells were γ-irradiated and resuspended in 500 µl phosphate-buffered saline (PBS); a 50-µl aliquot was assessed for isotope incorporation in a γ counter and the total injected radioactivity was calculated. Balb/c mice were injected i.p. (n = 10) and i.v. (n = 10) with labeled cells and sacrificed at different times after injection (2 mice per assay at 2, 8, 24, 48, and 72 h). All major organs were collected, weighed, and assessed for radioactivity in a γ counter. Results are expressed as cpm/g organ collected.

Healthy dogs

Three dogs, 4-month-old siblings bred at the School of Veterinary Medicine of the University of Pennsylvania (Philadelphia, Pa.), were used in this study. Two dogs were male, one was female and all weighed 9–11 kg. All three dogs received two doses of cyclosporin A (CsA, each dose 15 mg/kg, per os) as immunosuppressive agent to prevent rejection of the xenogeneic cells. One CsA dose was given 24 h before TALL-104 cell injection and one just before cell administration. The dogs were sedated with oxymorphone and injected i.v. with γ-irradiated TALL-104 cells (10^8/kg in 50 ml saline) by slow (30 min) infusion through a venous catheter positioned in a distal vein of the front leg. The dogs were carefully observed for signs of acute toxicity during the infusion and in the following month. Blood samples were taken at 0, 4, 8, 24, and 48 h after TALL-104 cell injection. Blood cell counts and a complete serum chemistry profile were performed (Table 2). All laboratory tests were repeated weekly for 1 month. At that time, dogs were euthanized with a lethal dose of pentobarbital (150 mg/kg) and a complete necropsy was performed.

Tumor-bearing dogs

Thirty dogs bearing spontaneous malignancies of various histological types (including lymphoma, breast cancer, malignant histiocytosis, nasopharyngeal squamous cell carcinoma, melanoma, mast cell tumor, hemangiosarcoma) were entered in the study.

Nineteen dogs had advanced refractory disease and 11 dogs were in clinical remission at the time of enrollment in the TALL-104 study. All treatments were discontinued at least 1 week before cell administration. Dogs were divided into three groups according to the injection schedule. In schedule I, γ-irradiated TALL-104 cells were administered every other day at a constant dose of 10^8/kg for 2 consecutive weeks followed by four weekly boosts. CsA was administered at a dose of 10 mg/kg p.o. daily, starting from the day before TALL-104 cell administration throughout the first 2 weeks of injections. In the following 4 weeks, CsA was given to the dogs only the day before and on the same day as the TALL-104 injection. In schedule II, CsA (5 mg/kg) was administered twice a day and irradiated TALL-104 cells (10^8/kg i.v.) were given daily for 5 consecutive days. In schedule III, CsA was withheld and irradiated TALL-104 cells (10^8/kg i.v.) were administered daily for 5 consecutive days followed by single monthly injections at the same dose. The number of cell infusions given to each dog varied from 4 to 17 and the total number of cells infused in each dog ranged from 4×10^9 to 10^{11}. Some of the dogs were hospitalized during the cell treatment but most were treated as outpatients. Clinical signs of acute toxicity (such as fever, chills, hypotension, diarrhea, vomiting, etc.) were monitored during and after each cell injection. For the dogs treated as outpatients the owners were properly instructed to report on the well-being of their pets during cell treatment. Complete cell counts and serum chemistries (Table 2) were performed on samples obtained from the dogs before the study and before each cell administration.

Non-human primates

Five young adult male cynomologus monkeys (3–6 years old) weighing between 3 kg and 6.3 kg were housed individually in a room air-conditioned to 25±2 °C with a relative humidity of 50%±20% at the Memorial Sloan-Kettering Cancer Center (New York, N.Y.). The animals were maintained on a 12-h light/dark cycle and were provided with commercial primate chow and water ad libitum; fruit was given daily. All of the animal experiments were conducted in compliance with GLP regulations for nonclinical laboratory studies issued by the United States FDA, with the Animal Welfare Act, and with the guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council. Prior to drug/cell infusion, the monkeys were surgically implanted with a Fogarty catheter through the jugular vein, to reach the right atrium,

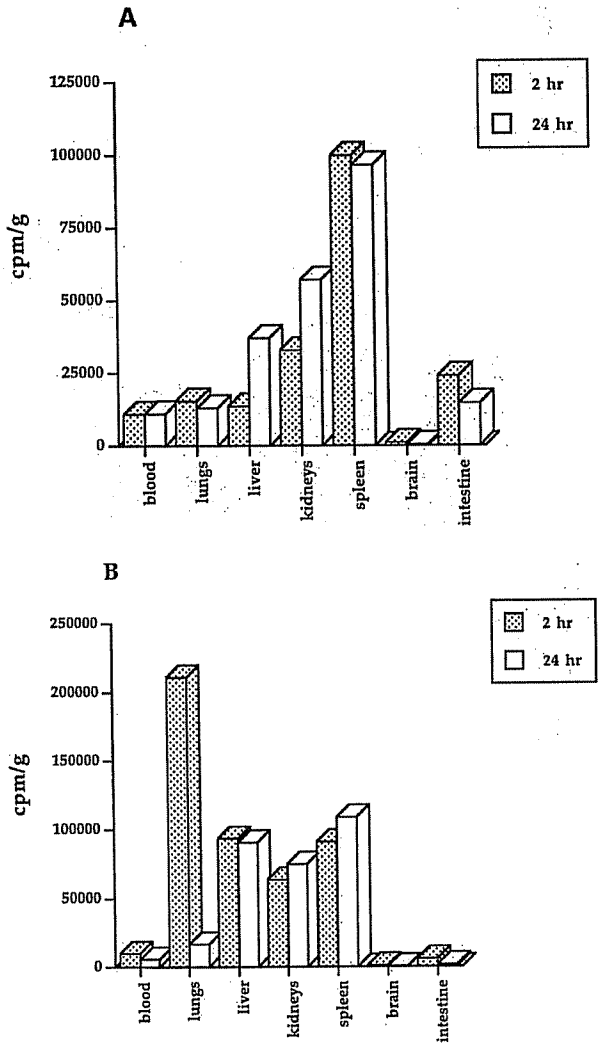


Fig. 1 Biodistribution of ^{51}Cr-labeled TALL-104 cells in mouse tissue. Balb/c mice were injected i.p. (**A**) or i.v. (**B**) with 2×10^7 ^{51}Cr-labeled TALL-104 cells. Mice were sacrified 2 and 24 h after injections, and lungs, livers, spleens, kidneys, brains, blood, and intestines removed, weighed, and assessed for radioactivity using a gamma scintillation counter

facilitating the repeated CsA and TALL-104 cell injections and blood sampling required by the experimental design. The doses of irradiated or unirradiated TALL-104 cells given and the schedules of cell injections and immunosuppressive therapy are detailed in Table 3. Clinical observations were recorded daily (before, during, and after each injection) and the animals were weighed weekly. Blood samples were withdrawn at different intervals for assessment of complete blood cell counts and serum chemistry profiles. Sera were also analyzed for the presence of human cytokines (see below).

Histopathology

Organs removed from mice (Balb/c and SCID) and healthy dogs at necropsy (including lungs, liver, spleen, kidneys, intestine, ovaries or testis, brain, lymph nodes, and spinal cord) were fixed in 10% buffered formalin (Fisher), paraffin-embedded, sectioned, and stained with hematoxylin/eosin for histopathological analysis.

Cytokine assays

Levels of human IFNγ, TNFα, TNFβ and granulocyte/macrophage-colony-stimulating factor (GM-CSF) were measured in serum samples obtained at different times before and after TALL-104 cell administrations (see above) using cytokine-specific enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Boston, Mass.), according to the manufacturer's procedure. The sensitivity of the assay was 20 pg/ml for IFNγ and TNFα, 8 pg/ml for TNFβ and 7.8 pg/ml for GM-CSF.

Immunological monitoring in dogs and monkeys

Serum and peripheral blood mononuclear cell (PBMC) samples [separated from whole blood by Accu-Prep (Accurate Chemical, Wesbury, N.Y.) lymphocyte gradient centrifugation] were obtained at different times before and after TALL-104 cell injections, and monitored for the development of humoral and cellular immune responses, respectively, against TALL-104 cells. Sera were diluted at 10^{-3} in fluorescence-activated cell sorting (FACS) buffer (Ca^{2+}- and Mg^{2+}-free PBS with 0.1% NaN_3 and 2% IgG-free horse serum) and incubated with TALL-104 cells (10^5/well) in a 96-well round-bottomed plate for 1 h at room temperature. After three washings in FACS buffer, a fluorescein-isothiocyanate-conjugated rabbit anti-dog IgG or anti-monkey IgG (whole molecule; Sigma) was added at 2×10^{-2} for 1 h at 4 °C. At the end of the incubation, cells were washed, resuspended in 150 µl FACS buffer, and analyzed by flow cytometry using an Ortho cytofluorograph cell sorter. The development of a TALL-104-specific cellular immune response was monitored in dogs and monkeys by testing the cytotoxic activity of the dogs' PBMC against ^{51}Cr-labeled TALL-104 cells in an 18-h ^{51}Cr-release assay [23]. MHC-non-restricted killing activity was measured in the same assays by testing dogs' PBMC against K562 cells.

PCR analysis

The presence and persistence of circulating TALL-104 cells in dogs and monkeys were evaluated by polymerase chain reaction (PCR) analysis of frozen aliquots of DNA extracted as described [32] from PBMC obtained at various intervals before and after TALL-104 cell injections. Two primers specific for the human minisatellite region YNZ.22 [32] were used. An oligonucleotide probe recognizing 24 nucleotides in the middle of the amplified sequence was used to demonstrate the specificity of the PCR products by Southern blot hybridization [32].

Results

Migration of i.v/i.p. injected TALL-104 cells in murine tissues

Radiolabeled, irradiated TALL-104 cells, injected i.p. into Balb/c mice, were detected mainly in the spleen within 2 h after injection (Fig. 1A) and accumulated also in the liver and kidneys 24 h later. The distribution of TALL-104-cell-associated radioactivity remained unchanged from 24 h to 72 h after transfer (not shown). Upon i.v. injection, most of the radioactivity at 2 h was localized in the lungs (Fig. 1B) whereas, after 24 h, TALL-104 cells were detected primar-

Table 4 Laboratory screens of healthy dogs injected i.v. with γ-irradiated TALL-104 cells (10^8/kg). *WBC* white blood cells, *PMN* polymorphonuclear leukocytes, ND not dance

Dog no.	Cells	Cell count (CC)		
		0 h	8 h	24 h
1	WBC	11100	17170	10400
	PMN	4551	10620	5720
	Lymphocytes	6105	6549	4160
	Monocytes	111	531	312
	Eosinophils	333	ND	208
2	WBC	12600	14700	12000
	PMN	7560	8820	6960
	Lymphocytes	4534	5733	4680
	Monocytes	504	147	240
	Eosinophils	ND	ND	120
3	WBC	11700	16400	10000
	PMN	7020	10660	5900
	Lymphocytes	4212	4100	3700
	Monocytes	117	820	200
	Eosinophils	351	192	200

ily in the liver, kidneys, and spleen, with much lower levels of radioactivity recovered from the lungs. Although the total recovery of radiolabeled TALL-104 cells was low after 48 h and 72 h (20%–40%), most of the detectable radioactivity was still in the liver and spleen (not shown). The same migration pattern was seen with non-irradiated TALL-104 cells (not shown) thus proving that irradiation does not affect motility or diapedesis of these cells.

Clinical observations in mice post-adoptive transfer

No clinical signs of acute toxicity were detected in any of the 180 SCID mice bearing human tumors (see Materials and methods) that were injected i.p. with different doses and schedules of administration of γ-irradiated TALL-104 cells (Table 1). Out of 80 tumor-bearing SCID mice successfully treated with 10^9/kg irradiated TALL-104 cells injected i.p. on alternate days for a total of 6 injections and weekly thereafter for a total of 6 weeks (Table 1), 25 were maintained for 1 year. These mice never developed any kind of delayed or chronic toxicity, including leukemia.

Clinical acute toxicity was noted in a group of 10 healthy Balb/c mice injected i.p. twice daily with irradiated TALL-104 cells at a dose of 5×10^9/kg and in a second group of 10 Balb/c mice treated with a single i.p. injection of 10^{10} cells/kg. Lethargy, ruffled fur, hunched posture and severe diarrhea with significant weight loss (from 23.1 ± 0.4 g to 17.8 ± 0.9 g mean weight) were observed within 24 h of the last TALL-104 injection in all mice. However, all symptoms, with the exception of weight loss, were transient and regressed completely in 48 h in 95% of the mice. One mouse died as a result of acute toxicity within 24 h of the cell infusion in the second group of mice treated with the highest dose. One week after the end of the injections the mean weight was 22.8 ± 0.4 g.

Table 5 Examples of leukocytosis with neutrophilia in tumor-bearing dogs before, during, and after TALL-104 therapy

Dog no.	White blood cells (l^{-1})	Granulocytes (%)
1	Before = 6.7×10^9	88
	During = 21.2×10^9	91 (highest value)
	After = 7.1×10^9	85
2	Before = 10.9×10^9	83
	During = $43.9.1\times10^9$	98 (highest value)
	After = 7.8×10^9	81
3	Before = 4.5×10^9	56
	During = 15.2×10^9	94 (highest value)
	After = 6.7×10^9	57
4	Before = 10.2×10^9	70
	During = 18×10^9	83 (highest value)
	After = 7.1×10^9	67

Clinical observations in dogs and primates

No acute toxicity was observed in the three healthy dogs after a single i.v. injection of irradiated TALL-104 cells (10^8/kg). In the 30 tumor-bearing dogs, no life-threatening acute reactions were observed during or after TALL-104 cell injections, except for an isolated reaction seen in a female dog with lung metastatic breast cancer upon the second TALL-104 cell injection. The dog collapsed 10 min after the start of the infusion, showing a weak pulse and decrease in capillary refill time. The infusion was immediately stopped, and the dog was treated with dexamethasone i.v. Once the dog was stabilized, the cell infusion was completed at a slower rate. After this episode, the dog was premedicated with dexamethasone before each TALL-104 cell injection with no further adverse reactions. Mild vomiting, responsive to antiemetics, and diarrhea were seen in one-third of the dogs.

No clinical signs of acute toxicity were detected in the four monkeys injected with non-irradiated TALL-104 cells during and after cell administration. Co-administration of OKT3 mAb (known to be a potent stimulus for lymphokine-release by TALL-104 cells) did not add any toxicity. The fifth monkey did not receive TALL-104 cells and was used as a control for immunosuppression-related toxicities (Table 3).

Hematological effects of adoptive transfer of TALL-104 cells

Transient (and in one case dramatic) alterations were seen in the results of the hematological tests of all 3 healthy dogs 8 h after the single TALL-104 cell infusion, consisting of an increase in white blood cell counts associated with an increase in the absolute number of granulocytes (Table 4). These alterations normalized within 24 h after infusion. Discrete leukocytosis with neutrophilia was also noted in about half of the tumor-bearing dogs: the number of white blood cells increased rapidly after the infusion, reaching 1.7–4 times the baseline levels with 83%–98% neutrophils

Table 6 Laboratory tests performed on blood samples of monkeys injected with TALL-104 cells. See Table 3 for schedule of cell administration for each monkey. *BUN* blood urea nitrogen, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *WBC* white blood cells, *PMN* polymorphonuclear leukocytes, *Lym* lymphocytes

Monkey no.	Test	Day 1	Day 4	Day 7	Day 14
1	BUN mg/dl	20	16	20	–
	ALT IU/l	153	137	128	–
	AST IU/l	134	92	78	–
	WBC×10³/cc	7.3	6.3	8.9	–
	PMN (%)	81	49	63	–
	Lym (%)	18	32	28	–
2	BUN mg/dl	10	29	12	–
	ALT IU/l	54	65	46	–
	WBC×10³/cc	10.4	10.4	13.7	–
	PMN (%)	18	21	12	–
	Lym (%)	76	77	86	–
3	BUN mg/dl	10	21	12	–
	ALT IU/l	39	47	41	–
	WBC×10³/cc	10.4	18.1	9.9	–
	PMN (%)	64	50	49	–
	Lym (%)	34	48	51	–
4	BUN mg/dl	21	18	14	–
	ALT IU/l	56	76	107	54
	AST IU/l	23	83	55	20
	WBC×10³/cc	12.6	25.8	19.6	13.1
	PMN (%)	57	58	58	54
	Lym (%)	45	36	43	43
5	BUN mg/dl	20	18	19	17
	ALT IU/l	58	58	58	56
	WBC×10³/cc	10.9	14.1	11.7	11.1
	PMN (%)	54	68	63	60
	Lym (%)	42	32	36	39

at 24 h, and returned to normal within 24–48 h after the last cell infusion (see examples in Table 5). No significant correlation was found between neutrophilia and schedule administration.

No significant hematological effects were observed on monkey no. 1, injected with 10^8/kg non-irradiated TALL-104 cells once a week for a total of two infusions, together with CsA (5 mg/kg twice daily), or in monkey no. 5 (control receiving only immunosuppressive drugs). However, the absolute number of white blood cells increased from 4 days after infusion in monkey no. 3 (who was injected with 2.5×10^8–5×10^8/kg non-irradiated TALL-104 cells on alternate days for a total of three injections together with CsA, (5 mg/kg twice daily), and in monkey no. 4 (who was injected with escalating doses of TALL-104 cells, ranging from 5×10^7–10^8/kg, daily for 7 days, together with 5 mg/kg CsA and methylprednisolone, 0.5 mg/kg twice daily). By day 7, this leukocytosis had resolved in monkey no. 3 but remained elevated in monkey no. 4 because of the different schedule of cell administration. The test normalized in this monkey 1 week after the last injection (Table 6).

Effects on serum parameters

Analysis of sera collected from Balb/c mice 24 h after the last injection showed increased liver transaminases, decreased total bilirubin and albumin, and hypokalemia and hyperchloremia (Table 7). These values normalized by day 7.

Chemistry profiles assayed on sera taken from healthy dogs 0, 4, 8, 24, and 48 h after the single TALL-104 cell injection and then weekly for 1 month revealed no abnormalities (not shown).

An increase in liver transaminases (highest alanine aminotransferase values, 439 IU; normal dog range = 0–77

Table 7 Serum chemistries on healthy immunocompetent Balb/c mice injected i.p. with TALL-104 cells (10^{10}/kg). All chemistry tests were run on pooled sera from 5 mice. *ALB* albumin, *TBil* total bilirubin

Test	0 h	24 h	7 days	Normal mouse range
ALT (IU/l)	46	180	39	28–132
ALB (g/dl)	3.74	2.41	3.61	2.5–4.8
TBil (mg/dl)	0.75	0.00	0.6	0.1–0.9
K^+ (mmol/l)	5.30	4.52	5.1	4.7–6.4
Cl^- (mmol/l)	111.6	128.7	121.1	92–120

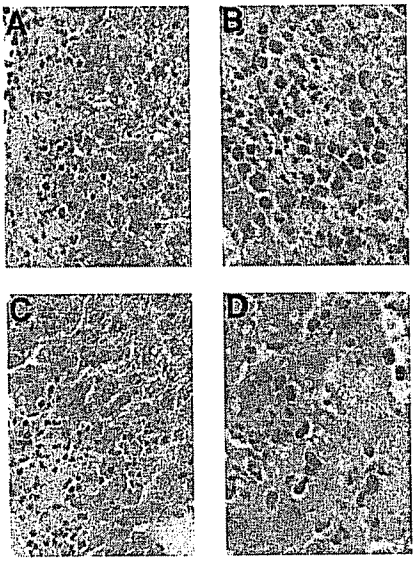


Fig. 2 A–D Histopathological analysis of livers from two representative Balb/c mice injected i.p. with 10^{10}/kg irradiated TALL-104 cells. Necrotic foci with lymphocytic/monocytic/neutrophilic infiltration are evident beneath the liver capsule. **A, B** Alterations at 24 h; **C, D** liver changes at 72 h. Hematoxylin/eosin staining; magnification 200× (**A, C**) and 400× (**B, D**)

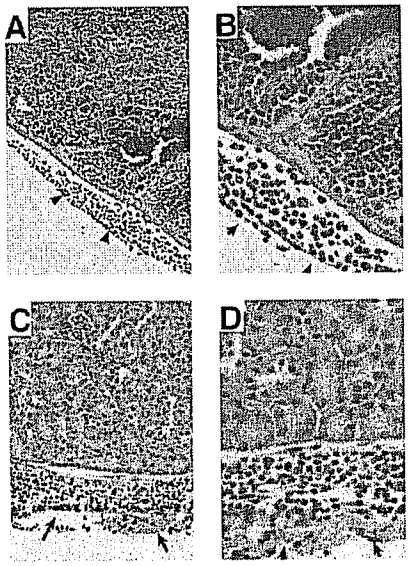


Fig. 3A–D Histopathological analysis of spleen (**A, B**) and kidney (**C, D**) from a representative Balb/c mouse injected i.p. with 10^{10}/kg irradiated TALL-104 cells 24 h before sacrifice. Note the presence of a layer of TALL-104 cells between the visceral peritoneum and the splenic capsule (*arrows*; **A, B**) or the renal capsule (*arrows*; **C, D**) but no infiltration into the organs. Magnification 200× (**A, C**) and 400× (**B, D**)

IU) was seen in fewer than 10% of the tumor-bearing dogs in the study, sometimes associated with a transient increase

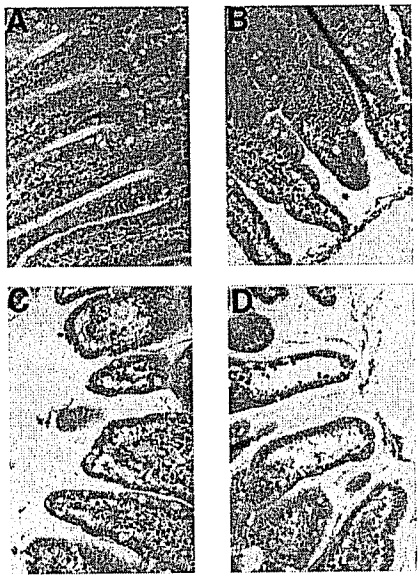


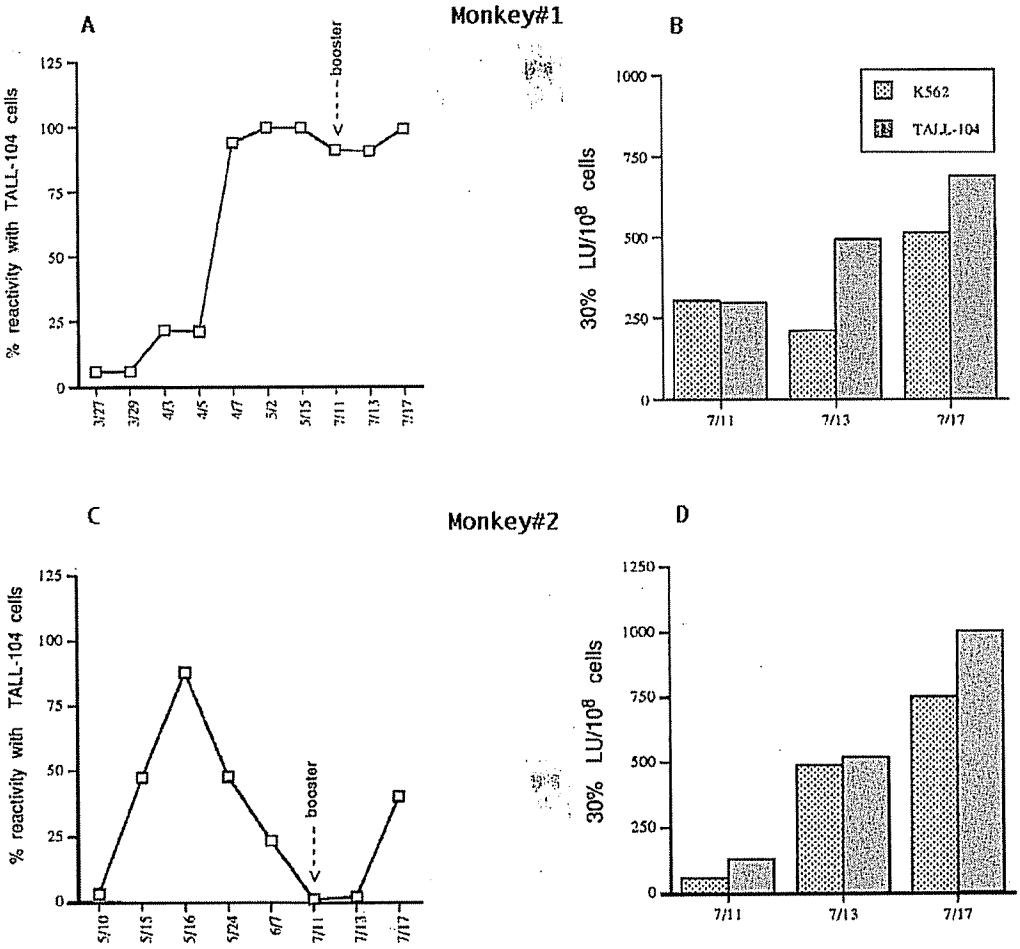
Fig. 4A–D Histopathological analysis of different segments of the intestine from a representative Balb/c mouse that developed diarrhea after i.p. injection of irradiated TALL-104 cells (10^{10}/kg). **A** Duodenum: despite the general increase in the number of muciparous cells in the villous epithelium, the structure of the villi is otherwise well preserved. **B** Illeum: modest alterations in some of the villi, limited to the luminal part. **C** Colon transversum: the alterations in the villi are more diffuse and severe. **D** Colon distale: dramatic destruction of most of the villous structure. All of these anatomopathological alterations were completely reversible within 72 h after TALL-104 cell injection. Hematoxylin/eosin staining; magnification 200×

in alkaline phosphatase (highest value, 1116 IU; normal dog range = 0–400 IU) and in total bilirubin (highest value, 4.41 mg/dl; normal dog range = 0–0.50 mg/dl). These alterations were transient and returned to normal levels as soon as cell injections were halted.

Small and transient increments in liver transaminases and blood urea nitrogen were noted in the sera of monkeys 2, 3 and 4, which nevertheless remained within the normal range (Table 6). These tests were back to normal by day 7 in monkey no. 2 and by 1 week after the last injection in monkey no. 4, because of the different schedule of cell administrations.

Macroscopic observations

Necropsy was performed on some of the SCID and Balb/c mice and in the 3 healthy dogs. No macroscopic findings appeared related to TALL-104 cell administration except for splenomegaly, which was noted in tumor-bearing SCID mice injected i.p. with 10^9/kg irradiated TALL-104 cells daily for 15 consecutive days or with 5×10^9/kg for 10 consecutive days. Notably, mice sacrificed within 2 weeks of the last cell injection had a significantly enlarged spleen (the mean spleen weight of untreated mice was 0.028 ± 0.005 g, whereas the mean spleen weight of treated mice was 0.102 ± 0.010 g) due, possibly, to the effects of



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cytokines (such as GM-CSF) produced by TALL-104 cells during tumor interaction and cross-reacting with lymphohematopoietic murine cells. Both histology and PCR analyses excluded the direct presence of TALL-104 cells in these spleens at that time (not shown).

Microscopic observations

The splenomegaly noted on macroscopic evaluation of tumor-bearing SCID mice receiving multiple injections of irradiated TALL-104 cells was found to be associated with an increase of granulocytopoiesis in the spleen (not shown). Otherwise, these mice showed no histopathological abnormalities other than those attributed to the implanted tumors. By contrast, Balb/c mice sacrificed 24 h and 72 h after the last i.p. injection had histological evidence of foci of hepatic necrosis with lymphocytic, monocytic, and granulocytic infiltration beneath the liver capsule and inside the parenchyma (Fig. 2). Subcapsular infiltrates of lymphoid cells (likely TALL-104 cells) were also detected in the spleens and kidneys (Fig. 3A, B and C, D respectively), but were never associated with necrosis and/or invasion of the parenchyma. The duodenum, ileum and rectum showed no significant histological abnormalities; however, dramatic histological changes were seen in the colon, consisting of an increased number of muciparous cells together with alterations in the structure of the villi (Fig. 4). The intestinal abnormalities were reversible (not detectable 72 h after the last cell injection), while the liver alterations were slower to repair, still being visible at 72 h (Fig. 2C, D). These observations were consistent with laboratory findings of persistent elevated transaminases at 48 h and 72 h, which slowly normalized over 1 week (see above).

Histopathological analysis of tissues obtained from the 3 healthy dogs sacrificed 1 month after a single TALL-104 cell injection demonstrated no organ toxicity (not shown).

Serum levels of human cytokines

No detectable levels of human (TALL-104 released) cytokines (TNFα, TNFβ, IFNγ, and GM-CSF) were found in

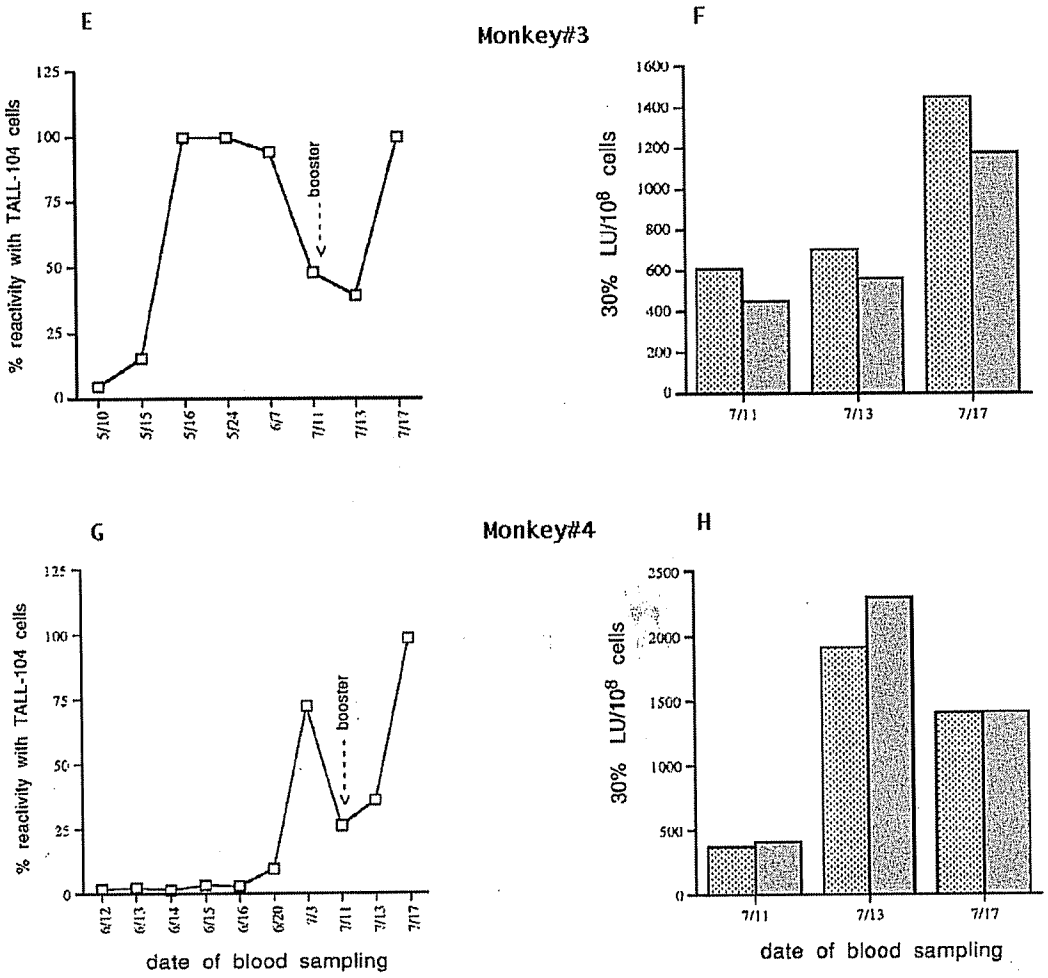

Fig. 5A–H Humoral (**A, C, E, G**) and cellular (**B, D, F, H**) immune responses against TALL-104 cells developed by 4 monkeys injected i.v. with different doses of non-irradiated TALL-104 cells (see Table 3 for dose and schedule of cell administration). Blood samples were obtained on the indicated dates. The presence of antibodies against TALL-104 cells was tested in monkey sera by fluorescence-activated cell sorting analysis as described in Materials and methods; monkey peripheral blood mononuclear cells (PBMC) were tested for cytotoxicity against K562 and TALL-104 cells in an 18-h ^{51}Cr-release assay

mouse sera (both SCID and Balb/c) collected 24 h after TALL-104 cell injection. However, significant levels of these cytokines were detected in the sera of some tumor-bearing dogs 24 h after TALL-104 cell injections (IFNγ, 30–120 pg/ml, TNFα, 25–132 pg/ml, and TFNβ, 40–150 pg/ml), but these levels were not associated with any clinical toxicity. Modest levels of TNFα and TNFβ (30–40 pg/ml) were also detected, although randomly, in monkey sera within a few hours after cell injection.

Immune response against TALL-104 cells

Despite the different immunosuppressive regimens with CsA given in association with steroids or not, virtually all healthy and tumor-bearing dogs (not shown) and monkeys (Fig. 5) developed a humoral immune response against TALL-104 cells, usually between days 8 and 12 after the first injection (Fig. 5A, C, E, G). Specific and aspecific cellular immune responses against TALL-104 and K562 cells (respectively) were demonstrated in 80% of the treated dogs (not shown) and in the monkeys (nos. 1–4) after boosting with γ-irradiated TALL-104 cells (Fig. 5B, D, F, H), but not in control monkey no. 5 (not shown).

Detection of circulating TALL-104 cells

PCR amplification of the human minisatellite region YNZ.22 was performed on PBMC isolated from the dogs and monkeys at different intervals before, during, and after cell therapy to document the time of appearance and the kinetics of disappearance of non-irradiated TALL-104 cells from the circulation. Circulating TALL-104 cells were always detectable in tumor-bearing dogs (not shown) and

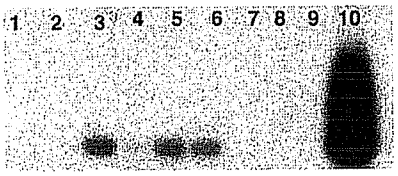

Fig. 6 Polymerase chain reaction amplification of the minisatellite region YNZ.22 performed on PBMC of monkey no. 1, obtained before and at different times after TALL-104 cell injections. *Lanes: 1* water (negative control); *2* time 0 first injection; *3* 4 days after; *4* time 0 second injection; *5* 4 h after; *6* 2 days later; *7* 4 days later; *8* 16 days later; *9* 4 months later; *10* TALL-104 cells (positive control)

in monkeys (Fig. 6) for a maximum of 1 week, irrespective of whether the TALL-104 cells were irradiated or not.

Discussion

The MHC-non-restricted cytotoxic TALL-104 cell line holds promise in future management of cancer as shown in several animal models with spontaneous and induced malignancies [29–31]. The present study was designed to investigate acute and long-term toxicities related to adoptive transfer of TALL-104 cells in different animal species using different routes and schedules of administration. The results show that, in the experimental conditions chosen, TALL-104 cell administration is a safe procedure not associated with major clinical toxicities. In fact, significant clinical toxicity was induced only in mice injected i.p. with very high doses of irradiated cells (approximately 100 times higher than the dose shown to have antitumor activity in vivo) [31]. The toxicity was limited to the gastrointestinal tract (diarrhea) and was completely reversible within 48 h of the last cell injection. Because such a large number of cells could be administered to the mice only i.p. and not systemically (without causing lethal pulmonary embolism), we cannot exclude the possibility that the gastrointestinal toxicity reflected local irritation of the intestine rather than an actual systemic toxic effect. In this regard, biodistribution studies in mice injected i.p. with radiolabeled TALL-104 cells (2.5×10^7/mouse) showed a high accumulation of radioactivity in the animals' intestines at 24 h (Fig. 2A). By contrast, gastrointestinal radioactivity was only marginal when the mice were injected i.v. with the same cell dose (Fig. 2B). Vomiting and diarrhea were also observed during i.v. TALL-104 cell administration in around 10% of tumor-bearing dogs, but these symptoms were always mild and easily controlled with appropriate therapy. The same gastrointestinal side-effects have been described in 80% of human patients during LAK/IL-2 therapy [33]. Abnormalities in liver function tests were observed in all species tested in this study, independent of the route and schedule of TALL-104 cell administration. However, in all cases, values returned to baseline 72 h to 1 week after the last cell injection, indicating that the insult to the liver was transient and reversible. Similarly, LAK/IL-2 therapy has been associated with altered hepatic functions [34], and it has been suggested that IL-2-activated lymphocytes are hepatotoxic; the strong correlation between peak lymphocyte counts after IL-2 priming and serum transaminase levels supports this contention [35]. As in the case of TALL-104 cells, the LAK-cell-induced liver abnormalities were transient and reversible.

None of the hematological abnormalities associated with IL-2/LAK therapy, including anemia, transient lymphopenia followed by rebound lymphocytosis, eosinophilia, and thrombocytopenia with coagulation disorder [36], were observed in the present study. However, various degrees of leukocytosis with relative neutrophilia were detected in both dogs and monkeys injected with TALL-104 cells. Some of the tumor-bearing dogs showed white blood cell counts two to five times above baseline levels in 24 h, with up to 99% neutrophils, perhaps reflecting the high levels of GM-CSF produced by TALL-104 cells in response to tumors [24]. However, we did not detect this cytokine in serum samples from TALL-104-treated animals at different intervals after cell administration, possibly because of (1) the time of sampling (24 h after cell injection might be too late to detect GM-CSF in the serum since secretion in vitro peaks at 8 h after the stimulus is applied); (2) the sensitivity threshold of our ELISA test (7.8 pg/ml may be insufficient); and/or (3) the fast metabolism and removal of the cytokine from the circulation. On the other hand, significant levels of IFNγ, TNFα, and TNFβ were detected in the sera of some tumor-bearing dogs and monkeys; however, no correlation was seen between these levels and clinical toxicity.

An important observation in the present study was the total absence, during TALL-104 cell injections, of clinical toxicities associated with increased capillary permeability, a finding that contrasts with preclinical and clinical experience with IL-2/LAK therapy [1–6]. The mechanisms by which IL-2/LAK cells induce vascular leak syndrome are unknown, but evidence suggests that this effect may be mediated directly or indirectly by host lymphoid elements activated by exogenous IL-2 administration [37]. Other data show that LAK cells can bind and lyse normal human vascular and corneal endothelial cells in vitro [38]. Although TALL-104 cells have not been tested for induction of vascular leak syndrome, this seems unlikely in light of the total absence of any signs associated with this syndrome in any of the animal species tested in this study. Moreover, TALL-104 cells maintain their antitumor activity in vivo without requiring exogenous IL-2 [29–31], so that administration of TALL-104 cells as single agent in prospective clinical trials should not be associated with the toxicity seen with LAK/IL-2 therapy.

The only serious acute toxicity observed in our study was an isolated reaction, easily controlled by steroids, observed in one tumor-bearing dog during her second consecutive injection with TALL-104 cells. This type of toxicity is to be expected in protocols involving injection of xenogeneic cells. The low incidence of toxic reactions in dogs and monkeys was even more surprising, considering that CsA (given to prevent TALL-104 cell rejection) did not block the development of either humoral and cellular immunity against TALL-104 cells.

Finally, the lack of circulating TALL-104 cells months after the last injections, as documented in the dogs by PCR analysis, and in mice, by histopathological analysis of tissues from animals sacrificed 1 year after the last injection with irradiated TALL-104 cells, rules out potential delayed sideieffects such as leukemia/lymphoma induced by irradiated TALL-104 cells. It is important to note that the same studies on monkeys demonstrated that even the non-irradiated TALL-104 cells are unable to proliferate, to induce sustained chimerism, or to induce leukemia in xenogeneic hosts.

Together, our data in animal models and the results from safety testing suggest that TALL-104 cells constitute a tumoricidal effector cell population that is relatively nontoxic against normal tissues in vivo. These studies, combined with previously reported preclinical studies, indicating the antitumor effects of irradiated TALL-104 cells in vitro and in vivo [29, 30], provide a basis for extending the evaluation of these cells to phase I and II trials in patients with TALL-104-sensitive tumors that are refractory to current chemo-radiotherapeutic regimens.

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X. RELATED PROCEEDINGS APPENDIX

None.